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Characterization of *Plasmodium* methionine metabolism key enzyme

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“There is inside you
All of the potential
To be whatever you want to be;
All of the energy
To do whatever you want to do.
Imagine yourself as you would like to be,
Doing what you want to do,
And each day, take one step
Towards your dream.
And though at times it may seem too difficult to continue,
Hold on to your dream.
One morning you will awake to find
That you are the person you dreamed of,
Doing what you wanted to do,
Simply because you had the courage
To believe in your potential
And to hold on to your dream.”

Donna Levine

ABSTRACT

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium* that are transmitted to humans by infected female *Anopheles* mosquitoes. Despite countless efforts toward eradication malaria still remains one of the most prevalent infectious diseases, constituting a major public health concern. The available antimalarial drugs are insufficient to control and eradicate malaria, mostly due to the emergence of drug-resistant parasites. Thus, the development of novel intervention strategies is critical to achieve eradication. As an obligatory intracellular pathogen, *Plasmodium* establishes close interactions with its host that are crucial to ensure parasite development and survival, one of such is the methionine metabolism. Methionine is an essential amino acid and, as for most living organisms, *Plasmodium* lacks the ability to synthesize methionine *de novo*. During the blood-stage of infection *Plasmodium* obtains methionine mainly through haemoglobin digestion. However, how *Plasmodium* obtains methionine during the liver-stage and how the parasite modulates the host cells in order to scavenge this essential amino acid is still unknown. The first step of methionine cycle is the synthesis of S-adenosylmethionine (SAMe) through a reaction catalyzed by the enzyme SAMe synthetase (SAMS). SAMe is a key metabolite in the methionine metabolism being the main biological donor of methyl groups for transmethylation reactions. SAMe is also a key intermediate in the transsulfuration pathway generating homocysteine (Hcy) which is metabolized into glutathione (GSH), being the last step of this pathway catalysed by glutathione synthetase (GS). GSH is a powerful antioxidant that in *Plasmodium* acts as one of the primary lines of the defense against the damage caused by reactive oxygen species (ROS), ensuring parasite survival. In this work we have explored the role of *Plasmodium* enzymes responsible for SAMe and GSH synthesis throughout its life cycle and in particular during the liver-stage of infection. The liver is a particular organ in the metabolism of methionine, namely in SAMe-dependent transmethylation reactions and in glutathione synthesis and storage. Thus, we hypothesized that while replicating inside hepatocytes, *Plasmodium* relies on its host to ensure a sufficient supply of these crucial metabolites. The data obtained in this study suggest that: 1) *Plasmodium* does not rely on its own SAMS enzyme while developing inside hepatocytes; 2) that the inhibition of SAMS activity during the blood-stage of infection leads to a low parasitemia, preventing the onset of cerebral malaria and 3) the deletion of GS-encoding gene results in the arrest at the oocyst stage, preventing transmission between the mosquito vector and the mammalian host. A detailed knowledge of *Plasmodium* methionine pathway provides promising tools for the design and development of novel antimalarial drugs.

Keywords: Malaria, *Plasmodium*, Host-pathogen interaction, Methionine metabolism, ROS

SUMÁRIO

A malária é uma doença causada por parasitas protozoários pertencentes ao género *Plasmodium* que são transmitidos aos humanos por mosquitos fêmea do género *Anopheles*. Apesar dos inúmeros esforços realizados na tentativa de erradicar a malária esta permanece ainda uma das doenças parasíticas mais prevalentes, constituindo um problema de saúde público. Os anti-maláricos disponíveis são insuficientes no controlo e erradicação da malária, devido sobretudo ao aparecimento de parasitas resistentes. Além disso, o escasso conhecimento acerca da biologia do parasita bem como das interações que este estabelece com o hospedeiro constituem uma barreira na luta contra a malária. Assim, o desenvolvimento de novas estratégias de intervenção torna-se crucial para conseguir a erradicação. *Plasmodium* é um patogénio intracelular obrigatório e, como tal, as interações que estabelece com o seu hospedeiro são essenciais para garantir o seu desenvolvimento e sobrevivência, nomeadamente as que estabelece ao nível do metabolismo da metionina. A metionina é um aminoácido essencial pelo que, tal como na maioria dos organismos, *Plasmodium* não tem capacidade para a sintetizar *de novo*. Durante a fase sanguínea *Plasmodium* obtém metionina maioritariamente através da degradação de hemoglobina. Contudo, os mecanismos que *Plasmodium* utiliza para obter metionina durante a fase hepática, bem como para modular a célula hospedeira de modo a garantir um fornecimento suficiente deste aminoácido são ainda desconhecidos. O primeiro passo do ciclo da metionina consiste na síntese de S-adenosilmetionina (SAMe) numa reação catalisada pela enzima SAMe sintetase (SAMS). A SAMe é um metabolito essencial na via metabólica da metionina sendo o maior dador biológico de grupos metilo. A SAMe é ainda um importante intermediário na via da transsulfuração sendo convertida em homocisteína e subsequentemente metabolizada em glutationo, sendo o último passo desta via catalisado pela glutationo sintetase (GS). O glutationo é um antioxidante que em *Plasmodium* atua como uma das primeiras linhas de defesa contra espécies oxidativas. Neste trabalho explorámos o papel das enzimas de *Plasmodium* responsáveis pela síntese de SAMe e glutationo ao longo do seu ciclo de vida, com particular ênfase na fase hepática da infeção. O fígado tem um papel preponderante no metabolismo da metionina, nomeadamente nas reações de transmetilação dependentes de SAMe bem como na regulação da síntese e armazenamento do glutationo. Assim, a hipótese que propusemos testar é que enquanto replica no interior do hepatócito *Plasmodium* depende do hospedeiro para garantir a obtenção destes metabolitos essenciais. Os resultados obtidos neste estudo demonstram que: 1) durante o seu desenvolvimento no fígado *Plasmodium* não depende da atividade da sua enzima SAMS; 2) a inibição da atividade da enzima SAMS durante a fase sanguínea da infeção resulta numa redução da parasitémia, prevenindo o aparecimento de malária cerebral e ainda que; 3) a deleção do gene que codifica para a enzima GS inibe o desenvolvimento dos esporozoítos, bloqueando assim a transmissão entre o vetor e o hospedeiro mamífero. Assim, um conhecimento detalhado do metabolismo da metionina em *Plasmodium* fornece ferramentas promissoras para o desenvolvimento de novos anti-maláricos.

Keywords: Malária, *Plasmodium*, Interação patogénio-hospedeiro, Metabolismo da metionina, ROS

RESUMO

A malária é a doença parasitária com maior impacto no mundo estimando-se que por ano 214 milhões de pessoas são infetadas, resultando numa taxa de mortalidade de aproximadamente meio milhão de pessoas. A África subsariana é a área geográfica com maior taxa de incidência contudo, a malária é também endémica no Sudoeste Asiático, na América Latina e ainda nas regiões Mediterrânicas Orientais. O agente infecioso causador de malária é um parasita do género *Plasmodium* cuja transmissão entre hospedeiros é mediada por mosquitos fêmea do género *Anopheles*. Em seres humanos existem 5 espécies de *Plasmodium* capazes de causar infeção: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* e *P. knowlesi*. Destas, *P. falciparum* é o parasita predominante em África sendo responsável pela manifestação mais severa da doença, a malária cerebral. Embora menos letal, *P.* vivax é a estirpe mais pandémica e caracteriza-se pela capacidade de gerar formas hepáticas dormentes, os hipnozoítos, que podem resultar em recidivas da doença. O ciclo de vida de *Plasmodium* é complexo, incluindo duas fases de desenvolvimento em hospedeiros distintos: o hospedeiro mamífero e o vetor, o mosquito *Anopheles*. O ciclo de vida de *Plasmodium* inicia-se quando um mosquito infetado se alimenta do sangue de um hospedeiro mamífero, depositando sob a sua pele centenas de esporozoítos. Os esporozoítos deslocam-se através da pele até entrarem num vaso sanguíneo migrando depois pela circulação em direção ao fígado onde, após atravessarem um determinado número de hepatócitos, estabelecem a fase hepática da infeção. Esta fase caracteriza-se por ser obrigatória e clinicamente silenciosa na qual o parasita se desenvolve rodeado por um vacúolo parasitóforo, denominado por forma exo-eritrocítica. No interior do hepatócito, o parasita inicia um ciclo de replicação assexuada denominado por esquizogonia no qual, cada parasita, origina milhares de merozoítos que são depois libertados na circulação sanguínea através de vesículas denominadas merossomas, iniciando assim a fase eritrocítica da infeção. Dentro do eritrócito os parasitas replicam mitoticamente desenvolvendo-se sequencialmente em anéis, trofozoítos e esquizontes. Os esquizontes diferenciam-se novamente em merozoítos levando à lise dos eritrócitos e consequentemente, ao aparecimento dos sintomas clínicos associados à doença. A nova descendência de merozoítos pode infetar novos eritrócitos, num ciclo que é reiniciado inúmeras vezes garantindo a progressão da infeção ou diferenciar-se nas formas sexuadas do parasita originando os gametócitos. Contudo, a divisão sexuada dos gametócitos apenas é possível no interior do mosquito. Assim, juntamente com a ingestão de sangue durante a picada do hospedeiro são também ingeridos os gametócitos. Estes migram até ao intestino médio onde se diferenciam em gâmetas masculino e feminino e sofrem fecundação, resultando na formação do zigoto. O zigoto sofre depois divisão meiótica adquirindo mobilidade sendo designado por oocineto, o qual migra pelo epitélio do intestino em direção à lamina basal onde se estabelece. Na lâmina basal o oocineto adquire uma forma globular, originando o oócisto. Este sofre um processo de maturação no qual se replica por mitoses sucessivas produzindo esporozoítos que, após rutura do oócisto, são libertados na hemolinfa. Os esporozoítos são formas móveis e alongadas, migrando depois em direção às glândulas salivares do mosquito onde estão aptos a infetar um novo hospedeiro, reiniciando assim o ciclo de vida.

Plasmodium tem uma capacidade replicativa notável atingindo taxas de crescimento enormes o que implica elevadas exigências nutricionais de modo a garantir a sobrevivência, nomeadamente de metionina. A metionina é um aminoácido essencial pelo que *Plasmodium* não tem capacidade para a sintetizar *de novo.* Sendo um parasita intracelular obrigatório, *Plasmodium* evoluiu no sentindo de assegurar as suas necessidades nutricionais derivando nutrientes a partir da célula hospedeira. Durante a fase sanguínea *Plasmodium* obtém aminoácidos maioritariamente através da degradação de hemoglobina da célula hospedeira. No entanto, esta contém apenas quantidades vestigiais de metionina não sendo suficiente para satisfazer as necessidades nutricionais do parasita. Assim, *Plasmodium* modula a célula hospedeira induzindo na sua superfície canais que asseguram a troca direta deste metabolito do citoplasma do eritrócito para o interior do parasita. Contudo, o modo como *Plasmodium* modula a célula hospedeira e em que medida é o crescimento do parasita dependente do hospedeiro durante a fase hepática não é ainda conhecido. Apesar de ser assintomática, esta fase caracteriza-se por um aumento exponencial no número de parasitas sendo também marcada por inúmeras interações estabelecidas entre parasita-hospedeiro, nomeadamente a nível metabólico. Este forte tropismo do parasita para o fígado humano em conjunto com uma correlação forte entre o metabolismo do hepatócito e o do parasita tornam a fase hepática da infeção de particular interesse para o desenvolvimento de novas estratégias que atuem antes do aparecimento dos sintomas clínicos associados à doença.

O primeiro passo do ciclo da metionina é catalisado pela enzima metionina adenosiltransferase (MAT) - também conhecida por S-adenosilmetionina sintetase (SAMS) - e consiste na síntese de S-adenosilmetionina (SAMe). A SAMe atua como co-substrato em inúmeras reações metabólicas sendo o metabolito percursor das vias de transmetilação, aminopropilação e transsulfuração. Após ceder os seus grupos metilo a moléculas aceitadoras como DNA, RNA, lípidos e proteínas a SAMe é convertida em S-adenosilhomocisteína (SAH) que é, por sua vez, metabolizada em homocisteína. A homocisteína é então sequencial- e irreversivelmente convertida em cisteína e glutationo, sendo a última reação desta via catalisada pela enzima glutationo sintetase (GS). Dada a arquitetura do hepatócito e o papel preponderante do fígado: 1) na metabolização da metionina; 2) na mediação das reações de transmetilação dependentes de SAMe; bem como na 3) síntese e armazenamento de glutationo procurou-se neste presente trabalho testar se enquanto replica no interior do hepatócito, *Plasmodium* recorre ao hospedeiro para garantir um fornecimento suficiente destes metabolitos subvertendo assim esta via metabólica da célula hospedeira para garantir a sua sobrevivência. Para tal, gerou-se um parasita transgénico recorrendo à técnica do domínio destabilizador na qual a expressão de SAMS no parasita é regulada de acordo com a presença, ou não, de trimetoprima (TMP). A capacidade de *Plasmodium* se replicar e de estabelecer infeção na ausência de SAMS foi avaliada durante o seu desenvolvimento tanto no mosquito *Anopheles* bem como no hospedeiro mamífero *in vivo,* em modelo de roedores e *in vitro,* na linha celular HepG2. Os resultados obtidos *in vivo* sugerem que na ausência de SAMS (*Pb*SAMS-DD – TMP) o desenvolvimento de *Plasmodium* durante a fase sanguínea é drasticamente afetado, resultando numa diminuição nos níveis de parasitémia. Além disso a ausência de SAMS leva a uma diminuição da virulência conferindo proteção contra a malária cerebral.

De seguida estudámos a importância de SAMS durante a fase hepática, *in vitro*. Os resultados obtidos demonstram que na ausência de SAMS os parasitas desenvolvem formas exo-eritrocíticas com igual tamanho e em igual número quando comparado com o grupo controlo (*Pb*SAMS-DD + TMP), sugerindo assim que esta proteína não é essencial para o desenvolvimento nem para a replicação de *Plasmodium* durante este estadio da infeção. Os resultados obtidos *in vivo* estão em concordância com os obtidos *in* vitro, reforçando que durante a fase hepática *Plasmodium* não depende da sua enzima para completar o desenvolvimento e para estabelecer com sucesso a infeção. Durante o desenvolvimento de *Plasmodium* no mosquito tanto o número de oócistos como o de esporozoítos produzidos por parasitas que não expressam SAMS são idênticos ao obtido para o grupo controlo, sugerindo assim que esta enzima não é essencial durante esta fase do desenvolvimento.

A infeção por *Plasmodium* induz no hospedeiro inúmeros mecanismos de defesa que resultam na produção excessiva de espécies reativas de oxigénio, que podem resultar na morte do parasita. Em contrapartida, numa tentativa de sobreviver, o parasita recorre às suas defesas nomeadamente a antioxidantes como o glutationo. Estudos anteriores demonstraram que, na ausência de y-GCS, a enzima limitante da reação de síntese de glutationo, *Plasmodium* não consegue completar o seu desenvolvimento nem estabelecer com sucesso a infeção no mosquito. Tal como referido anteriormente, a síntese de glutationo envolve duas reações sendo que a GS catalisa o último passo. Assim, os resultados previamente obtidos para y-GCS permitiram-nos prever que a deleção da proteína GS em *Plasmodium* não iria surtir qualquer efeito no desenvolvimento do parasita durante a fase sanguínea. Para testar a nossa hipótese, gerámos um parasita transgénico que não expressa o gene que codifica a enzima GS (*Pbgs*$^{-}$). Os resultados obtidos *in vivo* permitiram então comprovar que, durante a fase sanguínea a enzima GS não é essencial para o desenvolvimento de *Plasmodium*. Contudo, o desenvolvimento de *Plasmodium* no mosquito é completamente inibido na ausência de SAMS, sendo a síntese de esporozoítos completamente bloqueada. Em suma, o conhecimento detalhado da via da metionina poderá contribuir para o desenvolvimento de novos anti-maláricos e também de novas estratégias que bloqueiem a transmissão de *Plasmodium* entre o vetor e o hospedeiro mamífero.

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LIST OF ABBREVIATIONS

BSA: Bovine Serum Albumin

cDNA: Complementary DNA

DD: Destabilising Domain

DHFR: Dihydro-folate reductase

DMEM: Dublecco's Modified Eagle's Medium

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

EEF: Exoerythrocytic form

ϒ-GCS: ϒ- glutamylcysteine synthetase

GFP: Green fluorescent protein

GS: Glutathione synthetase

GSH: Glutathione

HA: Haemagglutinin

Hcy: Homocysteine

HRP: Horseradish peroxidase

HSP70: Heat shock protein 70

IFA: Immunofluorescence assay

***i.v.*:** Intra-venous

MAT: Methionine adenosyltransferase

NPPs: New permeability pathways

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffer saline

PBST: Phosphate buffer saline containing Tween 20

PCR: Polymerase chain reaction

PFA: Paraformaldehyde

PV: Parasitophorous vacuole

rRNA: Ribosomal RNA

RT-qPCR: Quantitative reverse transcription PCR

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT: Room Temperature

SAMe: S-adenosylmethionine

SAMS: S-adenosylmethionine synthetase

SDS: Sodium dodecyl sulphate

TMP: Trimethoprim

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I. INTRODUCTION

1. Malaria: a burden worldwide

Malaria is one of the most severe infectious disease in the world with an incidence of 214 million cases and a mortality rate of 438 000 individuals in 2015[1]. Despite several efforts for prevention and control, that greatly contributed to reduce the burden of the disease, malaria remains a public health concern. The vast majority of deaths occur in sub-Saharan Africa (90%) however, malaria is also endemic in South-East Asia, Latin America and Eastern Mediterranean regions[2]. Malaria is caused by intracellular protozoan parasites that belong to the genus *Plasmodium* of the phylum *Apicomplexan.* In humans, there are five species of *Plasmodium* that cause malaria, *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*. Among them, *P. falciparum* is the most virulent and lethal being responsible for the severe malaria syndromes, the fatal manifestations of the disease[3]. In contrast, *P. vivax* despite being less virulent is the most widely distributed having the ability to generate hypnozoites – dormant forms of the parasite - that can lead to relapses of the disease[4].

Plasmodium life cycle

Malaria parasites have a complex life cycle that relies on two obligatory hosts: the mosquito vector in which *Plasmodium* completes its sexual development, and the mammalian host where *Plasmodium* asexual development is completed (Figure 1.1).

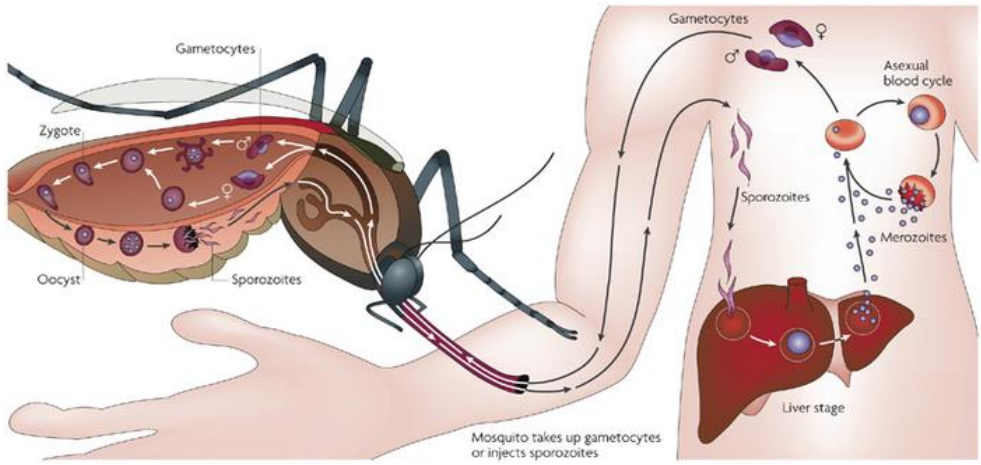

Figure 1.1 - *Plasmodium* life cycle: infection of the mammalian host and the mosquito vector. Infection of the mammalian host occurs when *Plasmodium* sporozoites are injected into the host skin by a female *Anopheles* mosquito, during a blood meal. Sporozoites enter the bloodstream and reach the liver, where each parasite invades a hepatocyte inside which grows and replicates into thousands of merozoites. Merozoites are then released in the bloodstream undergoing an asexual development inside the erythrocytes, a process that culminates with erythrocytes burst. Some parasites develop into sexual-stage gametocytes that can be ingested by a female mosquito during a blood meal. Inside the mosquito midgut, gametocytes develop to female and male gametes that fertilize and generate the zygote. In the lumen of the midgut, the zygote differentiates into the ookinete that migrates and egresses from the basal lamina. Ookinetes become sessile and develop into oocysts that grow extracellularly and burst, releasing thousands of sporozoites that migrate to the salivary glands. Sporozoites can be transmitted during the next mosquito blood meal restarting *Plasmodium* life cycle (Image from Su et al, 2007)[5].

The infection of the mammalian host occurs during a blood meal, when an infected female *Anopheles* mosquito injects the *Plasmodium* parasite in its sporozoite form under the host skin[6]. Sporozoites migrate through the dermis to enter a blood vessel and reach the liver, where they undergo an obligatory stage of development. Once in the liver sporozoites migrate through several cells before infecting a final hepatocyte[7]. This culminates with the development of a specialized compartment around the sporozoite, the parasitophorous vacuole (PV). The liver-stage of infection is clinically silent and leads to a 10000-fold increase in parasite numbers[8]. Inside the PV each sporozoite develops into an exoerythrocytic form (EEF) that grows as the parasite replicates. EFFs undergo several nuclear divisions by schizogony and differentiate into thousands of erythrocyte-infective merozoites that egress from hepatocytes into the bloodstream initiating the blood-stage of the infection[9,10]. Once in the blood merozoites invade erythrocytes undergoing an asexual developmental process in which, each merozoite subsequently develops through ring, trophozoite and schizont-stages. Schizonts replicate into up to 32 daughter merozoites (depending on *Plasmodium* spp.) culminating with erythrocyte burst. The released merozoites infect new erythrocytes ensuring the progression and expansion of infection. This process is repeated umpteen times being responsible for the onset and the clinical symptoms of the disease[11].

During the blood-stage of development triggered by specific environmental factors, a small subset of parasites enter a sexual phase enabling transmission from the mammalian host to the anopheline vector. In this sexual stage, parasites develop into male and female gametocytes that during a blood meal can be taken up by a female *Anopheles* mosquito. Within the mosquito midgut, triggered by lower temperature, higher pH levels and other mosquito-specific factors, gametocytes develop into gametes. Gametes will subsequently undergo fertilization leading to the formation of the zigote[12]. The zygote develops into motile and invasive forms called ookinetes that cross the midgut epithelial cell layer, from the apical side towards the basal lamina. This step results in a reduction in ookinete numbers, owing to vector immune response. Ookinetes that survive become sessile and differentiate into oocysts. Oocysts are stationary spherical structures being the only extracellular developmental stage of *Plasmodium* life cycle. Through several divisions each oocyst develops into thousands of sporozoites that migrate to the mosquito salivary glands. If a *Plasmodium*-infected female mosquito bites a new human host, sporozoites are injected under the skin restarting the life cycle[8].

2. Methionine: an essential amino acid to sustain life

Methionine is an essential amino acid for most living organisms, as such, there is no biosynthetic pathway for its *de novo* synthesis, meaning that it has to be obtained through the diet. As a sulphur-containing amino acid, methionine is crucial for cell survival. A sufficient supply of methionine through the diet has a massive importance for the normal growth and development of mammals[13]. Indeed, genetic defects or abnormalities in the methionine metabolism are associated with cardiovascular and liver disease and with cancer[14,15]. After digestion of dietary proteins methionine is distributed, through the blood, to all tissues and cells where it is used for protein synthesis or as a substrate. While all the mammalian cells have the ability to metabolize methionine, the liver is the central organ where about 50% of all dietary methionine is metabolized[16]. Methionine is the precursor of S-adenosylmethionine (SAMe), the main biological methyl group donor that is synthesized in the cytosol of every cell. SAMe is mostly consumed in methyl transfer reactions and has a key role in the methionine metabolism, being the precursor metabolite for transmethylation, aminopropylation and transsulfuration

pathways[17,18]. SAMe is a crucial regulator of multiple hepatic functions[19] as such, the maintenance of a constant hepatic content of SAMe regardless of the daily intake of methionine has an enormous importance for the normal function of the liver, being the amount of SAMe that is synthesized and catabolized strictly regulated, ensuring homeostasis[20].

2.1 Methionine Metabolism in Mammals

The metabolism of methionine in mammals consists in the transmethylation, the transsulfuration and the aminopropylation pathways (detailed in Figure 1.2). Methionine cycle starts with methionine conversion into SAMe, by the enzyme methionine adenosyltransferase (MAT) – also known as S-adenosylmethionine synthetase (SAMS) - using ATP as a cofactor. In mammals, two MAT isoenzymes were described: MAT I/III, encoded by the liver-specific *mat1a* gene and MAT II, expressed in all tissues and encoded by the *mat2a* gene[13]. As a methyl donor, SAMe transfers its methyl groups to acceptor molecules like DNA, RNA, proteins and phospholipids in a reaction catalyzed by specific methyltransferases (MTs). As a by-product, S-adenosylhomocysteine (SAH) is synthesized. SAH is a toxic intermediate that acts as a strong inhibitor of all transmethylation reactions by inhibiting MTs activity. The ratio between SAMe and SAH - methylation index – regulates the overall methylation capacity of the cell, being a sensitive indicator of cellular methylation status. In fact, a steep increase in SAH levels or decrease in SAMe levels can lead to disorders in the metabolism of methyl groups, which can consequently lead to cell death[17]. This means that the removal of excessive SAH is not only required but also critical for normal cell growth and survival.

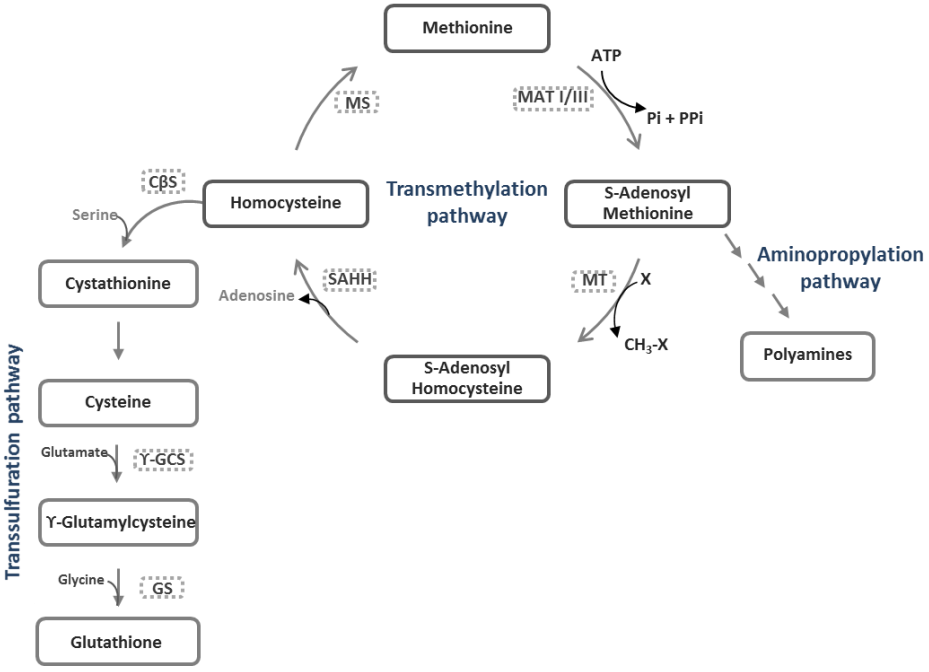

Figure 1.2 – Methionine metabolism in the mammalian liver. Methionine is converted to S-adenosylmethionine (SAMe) by methionine adenosyltransferase (MAT), using ATP as a co-substrate. SAMe can either be used to synthesize polyamines (aminopropylation pathway) or transfer its methyl groups to acceptor molecules leading to S-adenosylhomocysteine (SAH) synthesis (transmethylation). SAH is hydrolyzed to homocysteine (Hcy) and adenosine in a reversible reaction catalyzed by the SAH hydrolase (SAHH). Hcy can be either remethylated to salvage methionine or enter the transsulfuration pathway to be converted to cystathione and then to cysteine. Cysteine is then converted to Ɣ-glutamylcysteine (Ɣ-GC) and glutathione (GSH) in a two-step reaction catalyzed by Ɣ-GC synthetase (Ɣ-GCS) and GSH synthetase (GS), respectively.

SAH hydrolase (SAHH) is the only eukaryotic enzyme able to catalyze the reversible reaction in which SAH is hydrolyzed into adenosine and homocysteine (Hcy), completing the transmethylation pathway of the methionine metabolism[21]. Hcy occupies a branch point in the methionine cycle that according to biological requirements can be either re-methylated in order to regenerate methionine or irreversibly removed from methionine cycle, entering the transsulfuration pathway allowing for cysteine synthesis – the rate-limiting amino acid for glutathione (GSH) synthesis[16,22]. A condensation reaction between Hcy and serine, catalyzed by the enzyme cystathionine β-synthase (CβS), allows cystathionine synthesis. Cystathionine is then cleaved into cysteine that can be either used for protein synthesis or, due to its high reactivity, for GSH synthesis. GSH is synthesized in the cytosol of all mammalian cells in a tightly regulated manner. The synthesis rate of GSH is dependent on cysteine availability and in γ-glutamylcysteine synthetase (γ-GCS) activity – the rate-limiting enzyme - occurring via a two-step reaction[23]. The first step is catalyzed by the enzyme γ-GCS, allowing γ-glutamylcysteine synthesis and the second step by the enzyme GSH synthetase (GS), a condensation reaction between γ-glutamylcysteine and glycine that yields GSH[24]. While all mammalian cells are equipped with the enzymatic machinery that allows methionine cycle, the transsulfuration pathway has a more limited distribution in mammalian tissues.

2.2 Glutathione as a powerful tool in ROS detoxification

The aerobic metabolism implies the production of ROS thus, a source of reducing equivalents is required by all cells to ensure the maintenance of an appropriate intracellular redox environment. The redox state of a cell is evaluated by the ratios between reduced and oxidized pyridine nucleotides - $NAD(P)H/NAD(P)^+$ - and by the ratios between reduced and oxidized thiols, such as the ratio glutathione/glutathione disulphide - GSH/GSSG [25]. These ratios and the intracellular levels of reactive oxygen species (ROS) are linked to the extent that, the activity of the enzymes involved in antioxidant processes depends on the redox state of the cell[26]. ROS play vital roles in cell physiology since that are involved in life cycle regulation, induction of signaling pathways and, moreover, in the stimulation of antioxidant responses[27].

However, if these antioxidant mechanisms fail to counterbalance the production of these reactive species the redox balance is lost and an oxidative stress occurs. In order to strictly regulate intracellular ROS levels aerobes evolved efficient antioxidant systems, including enzymatic scavengers of ROS - like superoxide dismutases - non-enzymatic molecules such as vitamins - vitamin E (tocopherol) and vitamin C (ascorbate) - and peptides such as the tripeptide GSH. GSH is a tripeptide of glutamate, cysteine and glycine (γ-L-glutamyl-L-cysteinyl-glycine) being the most abundant low-molecular weight thiol in mammals. It is present in all mammalian tissues and cells, being particularly high concentrated in the liver[28]. In eukaryotic cells 90% of the cellular GSH is found in the cytoplasm but it can also be found, at much lower levels, in the mitochondria and in the endoplasmic reticulum. This tripeptide can be found in its thiol-reduced form (GSH) or in its disulfide-oxidized form (GSSG). However, intracellularly, glutathione is maintained in its reduced form by the enzyme GSSG reductase[29]. GSH is a crucial regulator of many cellular events including xenobiotics detoxification, modulation of cell proliferation, DNA and protein synthesis and it is also the most abundant endogenous antioxidant. As a powerful antioxidant GSH plays a pivotal role in the maintenance of the intracellular redox status. Furthermore, it has high stability and is a non-toxic compound which makes GSH an outstanding redox buffer[30].

3. Importance of methionine during *Plasmodium* infection

As a rapidly multiplying organism *Plasmodium* has high nutritional and metabolic demands including amino acids, one of such the essential amino acid methionine. Like most living organisms *Plasmodium* cannot synthesize methionine, but its genome encodes all the key genes necessary for methionine catabolism, one MAT enzyme, several putative methyl transferases (MT), a SAHH, a SAMe decarboxylase (SDC) essential in polyamines synthesis and a GSH synthetase (GS)[13]. During the intraerythrocytic stages malaria parasites can acquire amino acids for protein synthesis from: 1) degradation of host haemoglobin; 2) uptake of free amino acids present in the host plasma; or 3) through *de novo* synthesis[31]. Haemoglobin degradation occurs in a specialized acidic organelle - the food vacuole – and is the major source for amino acids acquisition during that stage of development. Once inside erythrocytes *Plasmodium* ingests and degrades up to 75% of the host cell haemoglobin into amino acids, detoxifying the toxic free haeme into haemozoin, a dark brown pigment[32]. Nevertheless, only less than 20% of these amino acids are used for parasite protein synthesis, being the majority exported to the extracellular medium[33]. Thus, not all parasite amino acid requirements are achieved through haemoglobin degradation. The amino acids isoleucine and methionine are absent, or at such low levels in human haemoglobin that an external source is required[34].

Mature erythrocytes are terminally differentiated cells that are unable to synthesize proteins *de novo,* hence erythrocytes nutritional demands are ensured by the presence of membrane transporters, namely the L-system[35]. The L-system is a nutrient transporter for apolar branched or aromatic amino acids, such as the essential amino acid methionine. However, in infected erythrocytes *Plasmodium* nutritional demands are not fulfilled by the narrow range and limited amount of amino acids transported by the L-system. To survive in this scarce environment *Plasmodium* parasites evolved mechanisms to modulate human erythrocytes permeability, in a way that the directly exchange of metabolites is allowed[31]. An outcome of this modulation is the induction of new permeability pathways (NPPs) in the membrane of the host erythrocyte (Figure 1.3). NPPs are endogenous dormant channels, characterized by their high permeability to low molecular weight solutes and also by their broad-specificity and low-affinity. In *Plasmodium*-infected erythrocytes methionine is incorporated by the L-system at similar amounts of that in uninfected erythrocytes[36]. However, there is an additional influx due to parasite-induced NPPs, which results in a 15-fold increase in the uptake of methionine by *Plasmodium*-infected erythrocytes[37]. The transport of neutral amino acids through these NPPs is unidirectional and is trans-stimulated by the opposite movement of other neutral amino acid. This putative transport ensures the uptake of amino acids that are absent in adult human haemoglobin and that are required for *Plasmodium* growth. However, this uptake implies the removal of amino acids that are easily obtained through haemoglobin degradation and whose excessive accumulation can lead to osmotic overload. This interplay between the host erythrocyte and the parasite for nutrient acquisition, energy production and waste removal is the basis of a successful infection[34].

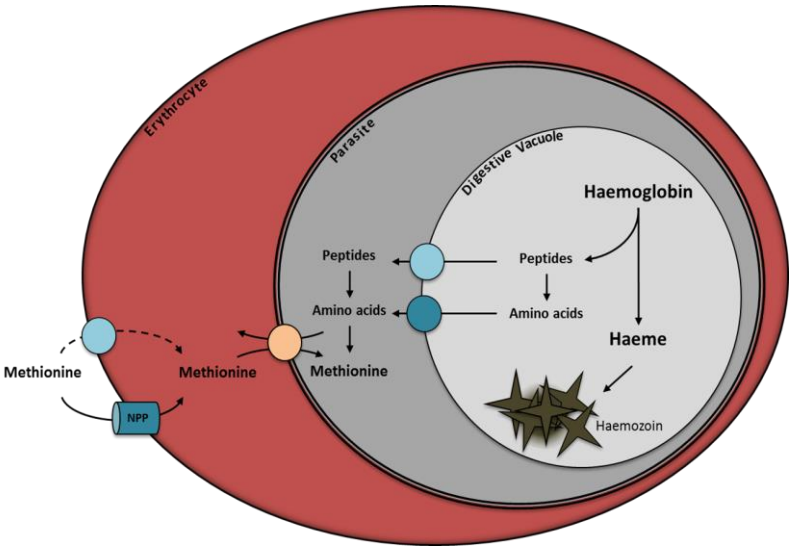

Figure 1.3 - Schematic representation of methionine transport in *Plasmodium*-infected erythrocyte. *Plasmodium* inability to obtain methionine from haemoglobin degradation within the host erythrocyte, is overtaken by the induction of new permeability pathways (NPPs) in the host cell membrane. This additional influx of methionine coupled with the amount of methionine that is transported by the L-system - an endogenous transporter of the erythrocyte - results in a 15-fold increase in methionine uptake by *Plasmodium*-infected erythrocytes (Image adapted from Cobbold et al, 2011)[34].

Much less is known about *Plasmodium* dependency on methionine during the other developmental stages of its life cycle. During the liver-stage each sporozoite generates a progeny of thousands of new parasites. Thus, the availability of large amounts of nutrients is mandatory for *Plasmodium* development and survival. Inside hepatocytes *Plasmodium* lives and replicates in a haemoglobin-free environment meaning that an alternative source is required[38]. However, the metabolism of methionine during *Plasmodium* liver-stage development remains elusive.

The interactions established between *Plasmodium* and the host inescapably lead to variations in the equilibrium between pro-oxidant and antioxidant molecules. This imbalance coupled with an excessive production of oxidizing species triggers an oxidative stress that can result in parasite death. The role of oxidative stress during malaria infection is still uncertain and some authors suggest a protective role, whereas others propose a close relation to the physiopathology of the disease[39]. Despite that, recent studies suggest that the excessive synthesis of ROS together with an oxidative stress play a pivotal role in the development of systemic complications associated to malaria[40]. *Plasmodium* infection inevitably leads to an increased oxidative stress both in the mammalian host as well as in the mosquito vector. In the mammalian host, oxidative stress occurs mostly due to: 1) the generation of hydroxyl radical ($OH^{\cdot}$) in the liver which can damage molecules like DNA, proteins and lipids leading consequently, to apoptosis[41]; and 2) due to the pro-oxidative environment of the erythrocyte. The erythrocyte has a high content of oxygen and iron, the key elements of the Fenton reaction - one of the major source of ROS production. Furthermore, the toxic free haeme that is released from digested haemoglobin inescapably leads to oxidation of Fe^{2+} into Fe^{3+} and to the release of superoxide anions, contributing also to an increased oxidative stress[26,40]. In fact, results obtained by Atamna et *al*. demonstrated that during the erythrocytic stage of infection, *P. falciparum*-infected erythrocytes produce twice more $OH^{\bullet}$ radicals and H_2O_2 when compared to normal erythrocytes[42]. During the mosquito-stage of development due to the innate immune response of the vector, *Plasmodium* is also exposed to a high oxidative stress mainly during ookinete and sporozoites development, which results in relevant parasite losses[26]. In order to survive and succeed in these oxidative environments, *Plasmodium* evolved efficient antioxidant systems namely superoxide dismutases and thioredoxin-dependent peroxidases. However, *Plasmodium* lacks catalase and glutathione peroxidases which is counterbalanced by the presence of a fully functional glutathione redox system[43].

II.AIMS

Plasmodium heavily depends on methionine to complete a successful development, as evidenced by a severe impairment in parasite development when perturbations in the methionine cycle, or in any of its branches (transsulfuration and aminopropylation) occur[44,45]. This dependence on methionine is more remarkable during the erythrocytic stages of *Plasmodium* life cycle, resulting in a 15-fold increase in methionine influx-rate into infected erythrocytes. Since there is a close connection between *Plasmodium* metabolism and the onset of the disease, unravelling the pathways and the mechanisms involved in the metabolism of this essential amino acid may be a rational and promising tool for developing both prophylactic agents and new chemotherapies against malaria[16]. Given the key role of the liver in: 1) methionine metabolism; 2) SAMe-dependent transmethylation reactions; and in 3) GSH synthesis and storage, the main aims of the present study focused the characterization of the importance of two main enzymes involved in the methionine metabolic pathway of *Plasmodium*: 1) GSH synthetase (GS) that is responsible for the *de* novo synthesis of glutathione and; 2) S-adenosylmethionine synthetase (SAMS) which allows SAMe synthesis, a crucial metabolite in the methionine cycle.

For this purpose, we will use the rodent malaria parasite *P. berghei* which, despite its phylogenetic distance, is analogous to human and primate malaria parasites sharing essential aspects as biology, physiology and life cycle. Additionally, *P. berghei* do not pose direct harm to humans and *in vivo* infected livers are directly accessible for analysis[46]. The availability of efficient reverse genetics technologies for *P. berghei* coupled with an ability to study and analyse its development throughout *Plasmodium* life cycle, make this species one of the greatest model to analyse parasite gene function[47,48]. Thus, we will characterize the role of *P. berghei* GS and SAMS enzyme during the mosquito-stage and in both the blood- and liver-stage *in vivo,* using rodent models and *in vitro,* using cell lines.

III. MATERIALS AND METHODS

1. Ethics Statement

All *in vivo* protocols were approved by the internal animal care committee of Instituto de Medicina Molecular and were performed according to the national and European regulations.

2. Mice and Parasites

Balb/c and C57BL/6J mice (6-8 weeks of age, female or male) were purchased from Charles River® Breeding Laboratories and housed in the Rodent Facility of IMM.
Wild-type *Plasmodium berghei* (*P. berghei*) ANKA strain was obtained from the *MR4* repository (Manassas, Virginia). Transgenic *P. berghei* parasite lines, namely GSH synthetase knockout (*Pbgs*$^{-}$), SAMS conditional knockdown (*Pb*SAMS-DD) and SAMS-green fluorescent protein (GFP) tagged (*Pb*SAMS-GFP), were generated in our laboratory. Asexual blood-stage parasitic forms were maintained through passage of infected blood in mice. All recombinant *P. berghei* parasite lines carry the human dihydrofolate reductase (*hdhfr*) gene cassete that confers resistance to pyrimethamine, allowing the selection of recombinant parasites. Confirmation of transgenic parasites genotype, construct integration at the desired genomic loci and elimination of WT locus were assessed by performing a polymerase chain reaction (PCR). To that end, blood from the infected mice tail vein was collected in 200µL of 1x PBS and genomic DNA (gDNA) was isolated using the NZY Blood gDNA Isolation Kit (NZYTech), according to manufacturer's guidelines. The PCR conditions used for genotyping were equivalent for all parasite lines and consisted of an initial denaturation at 95ºC for 10 min, followed by an amplification for 40 cycles of: 10 sec at 95ºC, 30 sec at 50 ºC and 1 min at 68 ºC. Amplification was followed by an additional extension step at 68 ºC for 10 min. Sequences of primers used for genotyping are provided in Table 1, Annexes section. Stabilisation of *Pb*SAMS-DD fusion protein throughout infection was achieved *in vivo,* by administration of trimethoprim (TMP) to mice (0.25 mg/ml of TMP in drinking water) and to mosquitoes (0.50 mg/mL of TMP in the mosquito food) or *in vitro,* to HepG2 hepatoma cells (0.5 or 5mg/mL of TMP in culture medium). The time of the treatment was set according to the experimental protocol.

3. Mosquitoes

Anopheles stephensi (*A. stephensi*) mosquitoes were obtained from the breeding facility at IMM and both housing and infections were performed at IMM. Mosquitoes were housed at 20ºC, 80% relative humidity and fed *ad libitum* with 10% glucose supplemented with 2% paraminobenzoic acid (PABA). Female mosquitoes were infected through feeding on mice infected with *P. berghei* ANKA WT, *Pbgs*$^{-}$*,* *Pb*SAMS-DD or *Pb*SAMS-GFP parasites.

3.1 Midguts dissection

To assess oocysts number and size, midguts were dissected in 1x PBS at day 12 or 16 (*Pbgs*$^{-}$) post mosquito infection and incubated with mercurochrome 0.1% (in H_2O) for 10 minutes. Stained midguts were washed in 1x PBS, mounted on glass slides and immediately analysed by light microscopy using the Leica DM2500 microscope (10x objective). To compare ROS levels, midguts were incubated with a

2 µM solution (in DMSO) of the general oxidative stress indicator 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA from Molecular Probes) for 20 minutes at room temperature (RT) under dark conditions. After incubation, midguts were rinsed in 1x PBS, mounted on a glass slide and immediately analysed by fluorescence microscopy using the Leica DM5000 B microscope (10x objective). Comparison of fluorescent levels between different conditions was performed as the same camera exposure times were used.

3.2 Salivary glands dissection

Sporozoites for infection were obtained by dissection of infected salivary glands 21 and 28 (*Pbgs*$^{-}$) days post mosquito infection. Salivary glands were dissected in Dulbecco's Modified Eagle's Medium (DMEM) and sporozoite number was assessed by light microscopy, using an improved Neubaeur cell counting chamber (depth 0.1 mm). Sporozoites were used for *in vitro* infection of HepG2 hepatoma cells or for *in vivo* infection of mice.

4. Parasite infections

4.1 *In vivo* infections

For liver-stage experiments mice were infected through intravenous (*i.v.*) retro-orbital injection of $5x10^4$ sporozoites while for blood-stage experiments mice were infected through *i.v.* injection of 10^3 sporozoites. To assess parasite liver load, livers were harvested 48 hours post-infection (hpi). To assess parasitemia levels in the blood throughout infection, thin blood smears fixed in 100% methanol were stained with Giemsa (10% v/v). Prepared slides were allowed to dry overnight before imaging and quantification was preformed using the Leica DM 2500 microscope (100 x oil objective)

4.2 *In vitro* infection

Liver-stage *in vitro* infections were performed in HepG2 hepatoma cells cultured in DMEM, supplemented with 10% fetal bovine serum, 2mM glutamine, 100 U/mL penicillin and streptomycin and maintained in a 5% CO_2 humidified incubator at 37^{o}C. For *P. berghei* infection, 6 X 10^4 HepG2 cells were platted (per well) in a 24-well culture plate and infection was performed 24 h post-seeding with 3 x 10^4 salivary gland sporozoites. Two hours after infection, culture medium was supplemented with 0.3% fungizone and infection was proceeded until suitable.

5. RNA isolation and analysis by RT-qPCR

Mosquito tissue: To isolate RNA from midguts and salivary glands, infected mosquitoes were dissected in 1x PBS supplemented with 1x Ribonuclease Inhibitors (NZY Tech). Tissue lysis was performed by adding 1mL of PureZOL RNA isolation reagent (BioRad®).
Mouse Blood: Blood from mice infected with synchronous parasites was collected at developmentally relevant time points post infection, by cardiac punction, allowing for isolation of RNA from ring- (6 hpi and 10 hpi), trophozoite- (14 hpi and 18 hpi) and schizont-stage (22 hpi) parasites. Infected blood was lysed by adding 1mL of TRIzol® LS Reagent (ThermoFisher scientific).

Mouse liver tissue: To extract RNA from mouse liver, whole livers were harvested 48 hpi in 1 mL of PureZOL RNA isolation reagent. Zirconia/Silica beads (1 mm) were added and tissue was disrupted using the Mini Bead Beater for 90 seconds.

Hepatoma cells: HepG2 cells were lysed at developmentally relevant time points post infection, through addition of 1mL of PureZOL RNA isolation reagent directly to the well.

For RNA extraction, chloroform was added to the lysates (240 µL chloroform per 1 mL of PureZOL/TRIzol) followed by vigorously vortex for 15 seconds and an incubation step at RT for 5 minutes. Samples were then centrifuged at 12000 x g for 15 minutes at 4°C allowing phase separation. The upper aqueous phase was collected and 100% ethanol (1.5x volume) was added to precipitate RNA. All subsequent steps were performed using the NZYtech Total RNA Isolation Kit, according to manufacturer's instructions. cDNA synthesis was performed using 1 µg of RNA and the NZY First-Strand cDNA Synthesis kit (NZYtech) according to manufacturer's instructions. PCR conditions used for cDNA synthesis were: 25°C for 10 min, 55°C for 30 min and 85°C for 5 min. Isolated cDNA was used to perform a quantitative reverse transcription-PCR (RT-qPCR) in a 10 µL reaction mix, containing 1 µL of sample cDNA. For each experimental condition negative controls were performed, either without RNA or reverse-transcriptase enzyme. For relative quantification of *Pbsams* expression levels, adenylosuccinate lyase (PBANKA_0304300) and serine-tRNA ligase (PBANKA_0615400**)** expression levels were used for normalization, whereas for infection load *Pb*18S rRNA levels were normalized against mouse hypoxanthine guanine phosphoribosyltransferase (*hprt*) housekeeping gene (ΔCt). The RT-qPCR conditions used consisted of an initial denaturation at 95°C for 5 min followed by amplification for 40 cycles at 95°C for 3 sec and at 60°C for 30 sec, with fluorescence acquisition at the end of each extension step. Gene expression values were then calculated based on the ΔΔCt method using the mean of control group as calibrator to which all other samples were compared. Primer pairs used to detect the target gene transcripts are listed in Table 1, Annexes section.

6. Immunofluorescence assays

6.1 *Pb*SAMS localisation *studies*

Mouse blood: For blood-stage experiments, blood smears from *Pb*SAMS-GFP-infected mice were fixed in 4% PFA (in PBS) for 10 min. Fixed cells were washed in 1x PBS and then permeabilized in 0.1 % Triton X-100 (in PBS) for 10 min. Blocking was then performed in 3% BSA (in PBS) for 30 min.

Hepatoma cells: For liver-stage experiments *in* vitro, HepG2 hepatoma cells cultured in coverslip were fixed in 4% PFA for 10 min, rinsed in 1x PBS and then permeabilized in 0.1% Triton X-100 for 10 min. After permeabilization, cells were blocked in 3% BSA for 30 minutes.

Mosquito tissue: For mosquito-stage experiments *Pb*SAMS-GFP-infected midguts and salivary glands were dissected in 1x PBS and DMEM, respectively. Midguts were fixed for 20min and salivary glands for 10min in 4% PFA. Fixed tissue was washed in 1x PBS, permeabilized in 0.1% Triton X-100 for 10 min and then blocked in 3% BSA for 30 min.

After blocking and permeabilization samples were incubated in the following primary antibodies: goat anti-*Pb*UIS4 (1:400, from Sicgen) and mouse anti-HSP70 (1:400, produced in house), for 2 hours at RT. The secondary antibodies used were: rabbit anti-GFP monoclonal antibody conjugated to Alexa Fluor 488 (1:400, from Thermo Fisher scientific), donkey anti-goat conjugated to Alexa Fluor 568 (1:400, from Life Technologies) and donkey anti-mouse conjugated to Alexa Fluor 647 (1:400, from Jackson ImmunoResearch Laboratories). DAPi (1:1000, from Sigma) was used for nuclear staining. Incubation with secondary antibodies was performed for 1 hour at RT. Samples were mounted with Fluoromount-

G™ (from Southern Biotech) and prepared slides were allowed to dry overnight before imaging. All images were obtained by fluorescence microscopy using the Zeiss Axio Observer microscope.

6.2 *Pb*SAMS-DD stabilisation by TMP treatment

For *Pb*SAMS-DD stabilisation verification, blood-stage and liver-stage *in vitro* samples were processed as described above (section 6.1) with exception of the antibodies employed for *Pb*SAMS detection. Additionally, *Pb*SAMS stabilisation during the liver-stage was also assessed *in vivo*. For this, the median liver lobe was collected and fixed in 4% PFA for 2 hours at RT, washed in 1x PBS and then sliced into 40-µm-thick sections using the Vibratome VT 1000S (Leica). Fixed lobes were blocked in 3% BSA and permeabilized in 0,3% Triton X-100 for 3 hours at RT. Samples were incubated overnight at 4ºC in the following primary antibodies: mouse anti-HA (1:400, from Covance), goat anti-*Pb*UIS4 (1:400, from Sicgen) and rabbit anti-*Pb*Bip (1:400, from Thermo Fisher scientific). The secondary antibodies used were: donkey anti-mouse conjugated to Alexa Fluor 488 (1:400, from Jackson Immonoresearch), donkey anti-goat conjugated to Alexa Fluor 568 (1:400, from Life Technologies) and donkey anti-rabbit conjugated to Alexa Fluor 647 (1:400, from Jackson ImmunoResearch Laboratories). For nuclei staining DAPi (1:1000 from Sigma) was used. Incubation with secondary antibodies was performed for 1 hour at RT. Samples were mounted with Fluoromount-G™ and prepared slides were allowed to dry overnight before imaging. All images of *Pb*SAMS-DD stabilisation were obtained by confocal microscopy using the Zeiss LSM 710 microscope.

7. Parasite pellet extraction for Immuno-blotting

All steps of parasite pellet extraction protocol were performed at 4º C to minimize protein degradation and all centrifugations were executed at 1000 x *g* for 10 min. Mice were sacrificed at day 10 post infection and 1mL of blood was collected by cardiac punction. The blood was washed in 10mL of 1x PBS and the pellet of packed erythrocytes was resuspended in 0.15% saponin and centrifuged. The parasite pellet obtained after lysis was washed in PBS containing 1x Proteinase inhibitor cocktail (Roche® cOmplete Protease inhibitor tablets, EDTA free). Parasite pellet was then resuspended in parasite pellet lysis buffer (4 % SDS; 0.5 % Triton X-114 in 1x PBS), incubated on ice for 10 min, centrifuged at 21000 x *g* for 10 min and the supernatant was collected. Total protein content was determined using the Bio-Rad protein assay kit according to manufacturer's instructions, as standards different concentrations of bovine serum albumin (BSA) were used. Protein samples diluted in 5x SDS sample buffer (NZYTech) were denatured at 95º for 10 min and resolved in an 8% polyacrylamide gel (SDS-PAGE). Proteins were blotted into a nitrocellulose membrane by wet transfer at 200mA for 2 hours. Primary antibody, mouse anti-HA antibody (1:1000, from Covance) was incubated overnight at 4ºC. Secondary antibody, anti-mouse horseradish peroxidase (HRP)-conjugated (1:10000, Santa Cruz Biotechnology) was incubated at RT for 1 hour. Signal detection was obtained using Luminata Crescendo Western HRP substrate (Merck Milipore®) and the ChemiDoc XRS+ Gel Imaging System (Bio-Rad®). Protein band quantification was performed on Image Lab software (version 5.0).

8. Statistical analysis

Statistically significant differences between two different groups were analysed using the Mann–Whitney test. $p < 0.05$ were considered statistically significant. Significances are represented in the figures as follows *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$. All statistic tests were performed using GraphPad Prism 5.0 software.

IV. RESULTS

Part I: Importance of *Plasmodium berghei* glutathione synthetase (*PbGS*) during the different stages of the life cycle

Plasmodium is a rapidly multiplying organism that undergoes a complex life cycle between two different hosts – a life style that entails a rapid adaptation to the external milieu. It is well described that during the different stages of the life cycle, *Plasmodium* is continuously exposed to high levels of ROS and oxidative stress. If not tightly regulated ROS can lead to deleterious effects resulting in parasite death. During the blood-stage of the infection the majority of ROS produced arise from haemoglobin digestion and free-haeme release within the PV of the parasite. Within the anopheline vector, *Plasmodium* is also exposed to massive amounts of ROS. Inside the midgut ookinetes survival is crucial for the establishment of a new parasite generation, for further production of sporozoites and consequent transmission to the mammalian host. The mosquito midgut is a harsh environment for *Plasmodium* and during that stage oxidative stress and ROS arise mostly from: 1) the innate immune response of the vector; 2) *Plasmodium* metabolic switching from glycolysis to oxidative phosphorylation for energy production, resulting in relevant parasite losses[49]. If produced in excessive amounts ROS are toxic and harmful to *Plasmodium*, being ookinete and sporozoite forms the most affected. To sustain life in this noxious environment, *Plasmodium* depends on its antioxidant defenses. In fact, *Plasmodium* is adapted to cope and withstand in this toxic environment as evidenced by its ability to fully develop within two completely different hosts[40]. One of *Plasmodium* primary lines of defense against these oxidative species is the tripeptide GSH. Thus, given the major role of GSH in ROS detoxification together with the high amounts of ROS that arise from infection, we decided to study the importance of glutathione synthetase (GS) during *Plasmodium* development.

1. Generation of a *Pbgs* knockout parasite line

To study the importance of GS during *Plasmodium* growth and development, GS-deficient *P. berghei* lines (*Pbgs*$^{-}$) were generated. The GS locus was disrupted by double crossover homologous recombination and the GS-encoding gene (*gs*) was deleted and replaced by the human dihydrofolate reductase (hDHFR) – a resistance cassete that allows for selection upon administration of pyrimethamine (Figure 4.1 A). Recombinant clones were obtained by limiting dilution of erythrocytes in a way that each mouse was infected with one single recombinant clone. Infected mice were treated with pyrimethamine in order to eliminate non-transgenic parasites *in vivo*. Mice parasitemia was assessed by counting GIEMSA-stained thin blood smears.

It has been described that knocking-out γ-GCS-encoding gene had no significant effect in growth and multiplication of blood-stage *Plasmodium* parasites*, in vivo*[50]. As γ-GCS is the enzyme upstream to GS in the transsulfuration pathway and the rate-limiting enzyme of glutathione synthesis, we predicted that the deletion of *gs* will not have any effect in *Plasmodium* development and survival during that stage of development.

We observed that wild-type (WT) and *Pbgs*$^{-}$ parasite multiplication rate was similar during parasite exponential growth in the blood-stage of infection (*data not shown*). Thus, the successful generation of transgenic *P. berghei* knockout lines (*Pbgs*$^{-}$) suggests that GS is not essential for the development of blood-stage parasites. Correct integration of the desired construct and the absence of WT parasites was confirmed by genotyping PCR (Figure 4.1 B). Two independent parasite clones, from two independent transfections were selected.

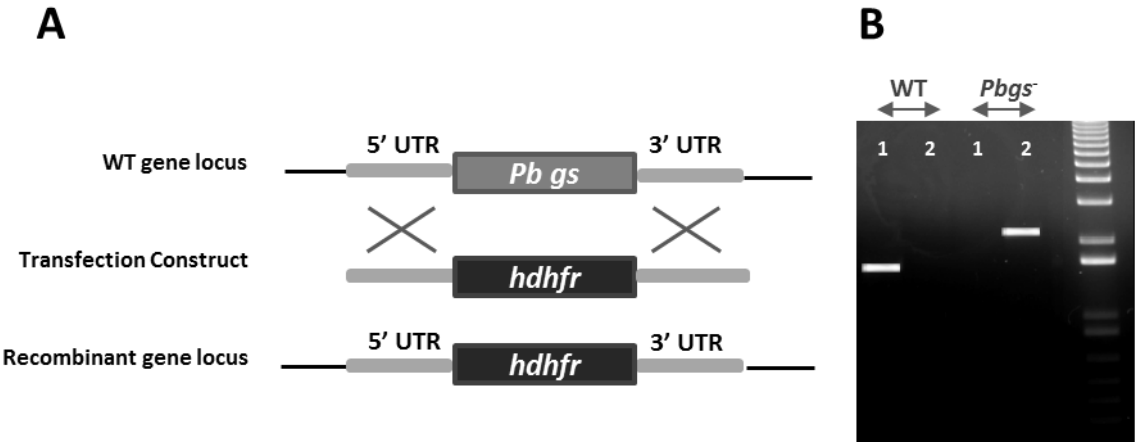

Figure 4.1– *Pbgs* is not essential during the blood-stage of infection allowing for gene deletion. (A) Double crossover recombination strategy for generation of a *P. berghei gs* knockout line (*Pbgs*$^{-}$). **(B)** Genotyping PCR confirming a clonal population of *Pbgs*$^{-}$ parasites. Lane 1: detection of WT *Pbgs* locus; Lane 2: detection of knockout construct integration locus.

2. GS is essential during the mosquito-stage development of *Plasmodium*

We then proceed with the characterization of GS function during *Plasmodium* development within the mosquito vector. For that purpose, *Anopheles stephensi* mosquitoes were infected with either WT or *Pbgs*$^{-}$ parasites. To evaluate the importance of GS in oocysts development, midguts were dissected 12 and 16 days post mosquito infection. Midguts were stained with mercurochrome and oocyst number was determined by light microscopy. Results obtained show that *gs* deletion resulted in a decrease in the number of *Pbgs*$^{-}$ oocysts in comparison to WT parasites (Figure 4.2 A). In addition, analysis of mercurochrome stained oocysts showed a marked reduction in *Pbgs*$^{-}$ oocysts size (Figure 4.2 B). To evaluate the ability of *Pbgs*$^{-}$ oocysts to develop and produce viable sporozoites, salivary glands were dissected at days 21 and 28 post mosquito infection. The results obtained show that sporozoites (spz) formation is completely impaired in *Pbgs*$^{-}$ parasites (WT spz number = 52 000 ± 8 000; *Pbgs*$^{-}$ spz number = 0; N=3). Thus, oocyst developmental arrest caused by the lack of GS results in an impairment in sporozoites formation.

Plasmodium is exposed to high levels of ROS during the mosquito-stage being the tripeptide GSH crucial in ROS detoxification and therefore, in the maintenance of the redox equilibrium. Thus, we hypothesized that the absence of viable oocysts and sporozoites may result from *Pbgs*$^{-}$ parasites inability to synthesize GSH and consequently, to detoxify ROS.

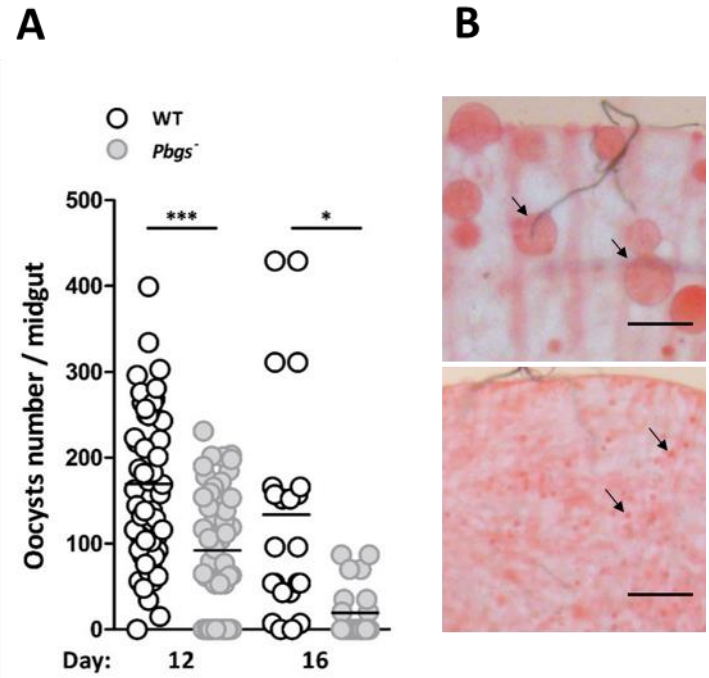

Figure 4.2– *Pb*GS is essential for *Plasmodium* oocyst development and sporozoite production. (A) Quantification of oocyst number in WT- or *Pbgs*-infected midguts stained with mercurochrome and imaged at day 12 and 16 post infection, using a light microscope. Each symbol represents an individual mosquito midgut. Horizontal lines represent the mean number of oocysts. Statistical significance was determined by the Mann Whitney U test, *p <0.05, ***p<0.001. **(B)** Representative pictures of oocysts size in midguts from WT- (upper) and *Pbgs*- (bottom) infected mosquitoes, stained with mercurochrome at day 12 dpi and imaged using a light microscope. Arrows indicate oocysts. Scale bar - 50µm. N=3

It has been described that blood meal-derived haeme leads to a decrease in ROS levels within the midgut of *Aedes aegypti* mosquitoes[51]. To test the effect of a blood meal on ROS levels in *Anopheles stephensi* midgut we analysed ROS levels in female mosquitoes fed with 10% glucose (sugar) or with blood. For this purpose, non-infected midguts from sugar- and blood-fed mosquitoes were dissected and incubated for 20 minutes in a 2µM solution of the oxidant sensitive probe 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy – H2DCFDA). Carboxy-H2DCFDA is a chemically reduced, acetylated form of fluorescein that is rapidly converted to a green-fluorescent molecule when its acetate groups are removed by intracellular esterases, resulting in an increased oxidation through ROS activity, within cells (Figure 4.3 A-C). Midguts integrity and the presence of blood in blood-fed midguts were confirmed by light microscopy (Figure 4.3 A) and ROS levels were analysed by fluorescence microscopy. The images show a strong CM-H2DCFDA fluorescence signal in midguts from sugar-fed when compared with blood-fed mosquitoes, indicative of high ROS levels (Figure 4.3 B).

Then we tested if decreasing ROS levels within *Pbgs*-infected midguts would rescue the oocyst development phenotype, allowing for *Pb*gs- oocysts full maturation and further differentiation in sporozoites. To that end, *Anopheles stephensi* female mosquitoes were fed either with sugar or with blood every 48 hours, until the end of the experiment. Midguts were dissected at day 12 post mosquito infection and stained with mercurochrome for further evaluation of oocyst number and size by light microscopy. Results obtained show that the decrease in ROS levels by blood-feeding do not rescue the oocyst developmental arrest phenotype. Blood-feeding of *Pbgs*-infected female mosquitoes had no significant effect in oocysts development both in size as in number, when compared to the sugar-fed control (Figure 4.3 C, D).

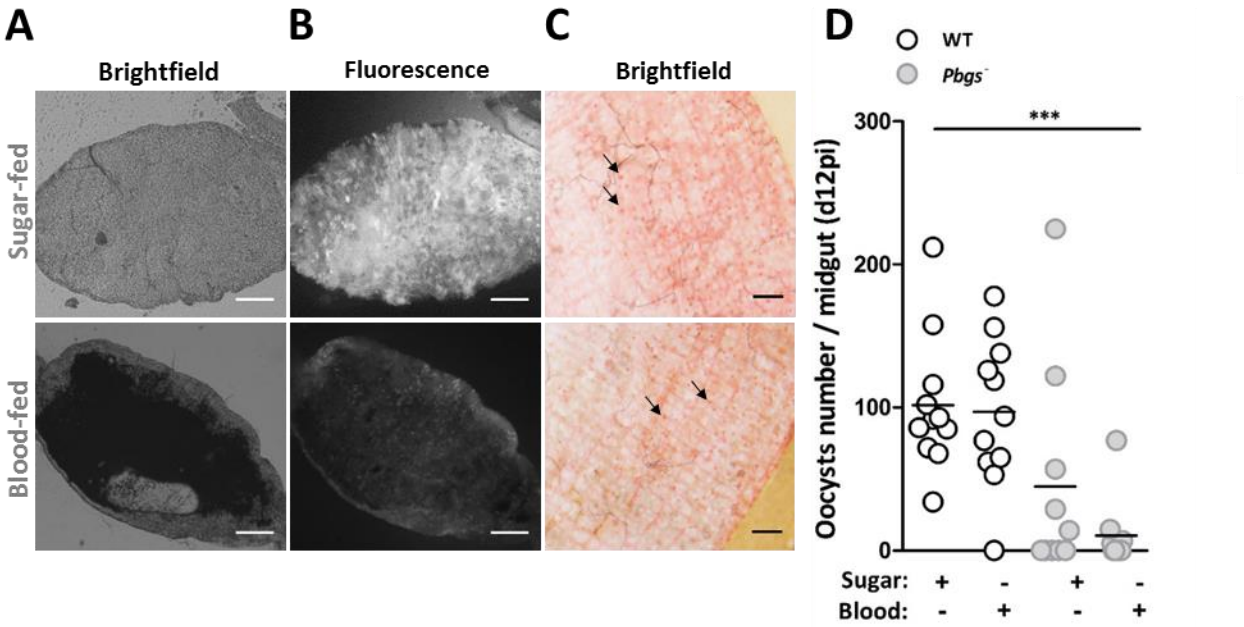

Figure 4.3 – Inhibition of ROS production in the midgut does not rescue oocyst developmental arrest in *Pbgs*⁻ parasites. **(A-C)** Representative pictures of sugar-fed (upper panels) or blood-fed (bottom panels) mosquito midguts 48 hours after the blood meal. **(A)** Midgut integrity and presence of blood in the lumen of blood-fed midguts assessed by light microscopy. Scale bar - 50µm. **(B)** Non-infected midguts from sugar- and blood-fed mosquitoes analysed for ROS levels through staining with the oxidant-sensitive probe carboxy-H2DCFDA (2µM) and analysed by fluorescence microscopy. Scale bar - 50µm **(C)** Representative picture of oocysts size in *Pbgs*⁻infected midguts from sugar- and blood-fed mosquitoes stained with mercurochrome and imaged by light microscopy at day 12 post infection. Scale bar - 100µm **(D)** Quantification of oocyst number in WT- or *Pbgs*⁻-infected midguts from sugar- and blood-fed mosquitoes stained with mercurochrome and imaged at day 12 post infection, using a light microscope. Each symbol represents an individual mosquito midgut. Horizontal lines represent the mean number of oocysts. Statistical significance was determined by the Mann Whitney U test, ***p<0.001. N=3

Salivary glands were dissected 21 post infection for assessment of sporozoite production. As before, no sporozoites were obtained both in sugar- or in blood-fed *Pbgs*⁻ infected mosquitoes (*data not shown*). Altogether, the results obtained show that the absence of glutathione leads to an impairment in *Plasmodium* development during the mosquito-stage, being *Pbgs*⁻ development completely blocked at the oocyst stage. The consequent inability to produce sporozoites compromises the transmission from the mosquito vector to the mammalian host, preventing further characterization of the role of GS during the liver-stage of development. Overall, these results highlight the importance of GS and consequently, GSH in the development and establishment of a successful *Plasmodium* transmission between the two hosts.

Part II: Importance of *Plasmodium berghei* SAMS enzyme during the different stages of the life cycle

1. Expression and localisation of *Pb*SAMS during the different developmental stages of *Plasmodium* berghei life cycle

To characterize the importance of *Plasmodium berghei* SAMS enzyme (*Pb*SAMS) in *Plasmodium* growth and replication we started by analysing the expression - both at mRNA and protein level - and localisation of *Pb*SAMS during the liver-, mosquito- and blood-stage of *Plasmodium* infection. Analysis of *Pbsams* relative gene expression during *Plasmodium* life cycle was performed by RT-qPCR using specific primers to *Pb*sams. To assess *Pbsams* expression during the blood-stage of infection a RT-qPCR was performed on cDNA synthesized from total RNA, extracted from blood infected with tightly synchronized parasites available in the laboratory. Gene expression was analysed during ring- (4hpi and 10hpi), trophozoite- (14hpi and 18hpi) and schizont- (22hpi) stage parasites development. The results show that *Pb*SAMS mRNA levels are higher immediately after invasion of erythrocytes during ring-stage parasites development, decreasing during development into trophozoite-stage to later increase during its differentiation into schizonts (Figure 4.4 A).

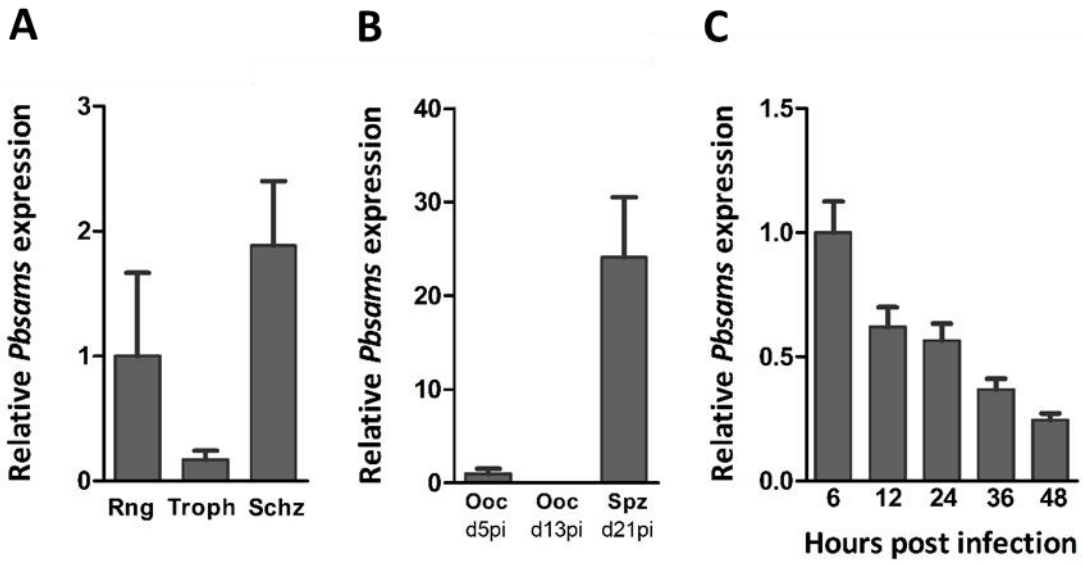

Figure 4.4 –*Pbsams* mRNA is expressed throughout all stages of *P. berghei* life cycle. Quantification of *Pbsams* mRNA by RT-qPCR **(A)** in blood-stage parasites: rings, trophozoites and schizonts; **(B)** in the mosquito-stage parasites: young-oocysts (day 5pi), mature-oocysts (day 13pi) and salivary glands sporozoites (day 21pi) and **(C)** in the liver-stage at different times post HepG2 infection with *P. berghei* ANKA sporozoites. *Pbsams* expression levels were normalized against two *P. berghei* housekeeping genes, adenylosuccinate lyase and serine-tRNA ligase. Error bars represent SEM. Rng: Ring; Troph: Trophozoite; Schz: Schizont; Ooc: Oocysts; Spz: Sporozoites. N=3

To extend investigation of *Pbsams* expression to the mosquito-stage, *P. berghei* -infected midguts and salivary glands were isolated. A RT-qPCR was performed allowing for quantification of *Pbsams* transcripts in young- and mature- midgut oocysts dissected at day 5 and 13 post infection, respectively and salivary gland sporozoites dissected at day 21 post infection.

Data obtained show that *Pbsams* has a low expression during oocysts development (young- and mature- oocysts) however, transcription of *Pbsams* gene increases 25-fold during sporozoites development (Figure 4.4 B). Lastly, for assessment of *Pbsams* expression during the liver-stage of infection, HepG2 hepatoma cells were infected with *P. berghei* sporozoites and isolated at relevant developmental time points post infection. The results show that expression of *Pbsams* mRNA is higher immediately after sporozoites invasion of HepG2 cells (6hpi) and decreases as intra-hepatic development progresses through sporozoite transformation (12hpi), replication (24 and 36hpi) and merozoite formation (48hpi; Figure 4.4 C). Altogether, the results obtained show that *Pbsams* is constitutively expressed through all the life cycle, being transcription levels particularly high in sporozoites.

Protein localisation studies were performed by using a previously generated transgenic *P*. *berghei* line, expressing a C-terminal green fluorescent fusion protein of *Pb*SAMS (*Pb*SAMS-GFP). *Pb*SAMS localisation during the different erythrocytic stages was assessed by immunofluorescence assay (IFA) of *Pb*SAMS-GFP-infected blood smears, using the anti-GFP antibody. Further analysis by fluorescence microscopy showed that *Pb*SAMS is expressed during all stages of erythrocytic development, exhibiting cytolosolic distribution and partially co-localising with *Plasmodium* endoplasmic reticulum protein Bip (*Pb*Bip; Figure 4.5).

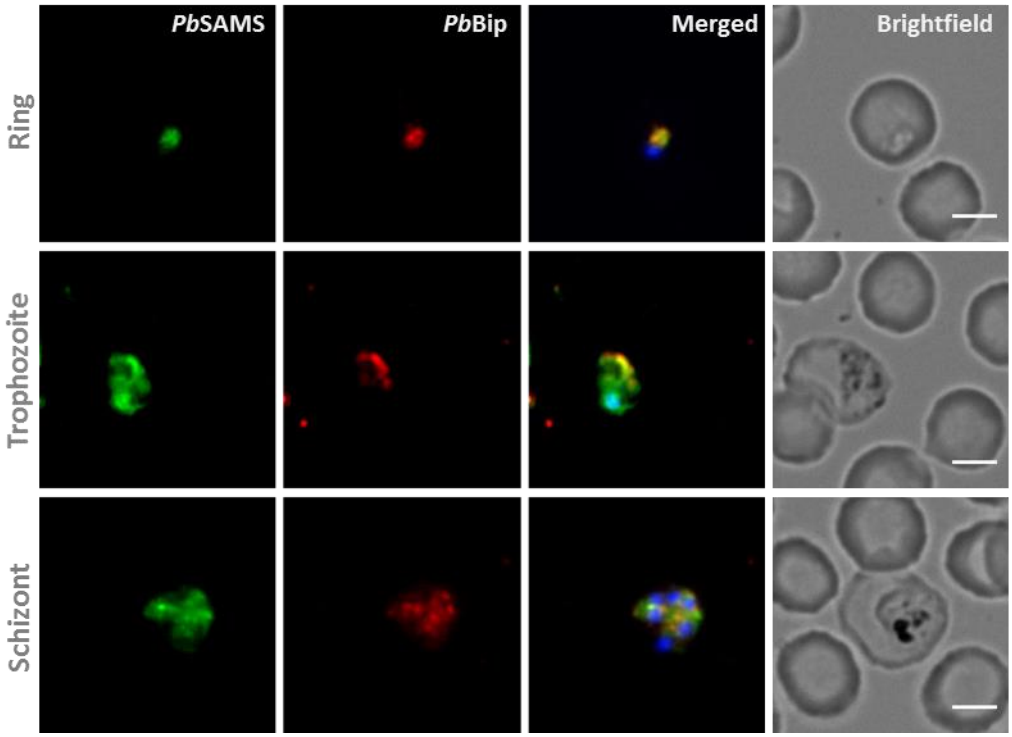

Figure 4.5– *Pb*SAMS is a cytoplasmic protein that partially co-localises with *Plasmodium* endoplasmic reticulum protein Bip during the blood-stage of infection. Widefield microscopy images of blood-stage *Pb*SAMS-GFP parasites, rings, trophozoites and schizonts, stained with the anti-GFP antibody for detection of SAMS-GFP protein and anti-Bip antibody for endoplasmic reticulum detection. DAPI was used for nuclei staining. Scale bar - 5 µm

*Pb*SAMS localisation was then investigated during the mosquito-stage, both in oocysts and sporozoites. *Pb*SAMS-GFP-infected mosquito midguts and salivary glands were dissected at days 12 and 21 post mosquito infection, respectively. Fluorescence microscopy analysis showed that during oocysts and sporozoites development, *Pb*SAMS co-localises with the *P. berghei* heat-shock protein 70 (*Pb*HSP70) to the parasite cytoplasm (Figure 4.6).

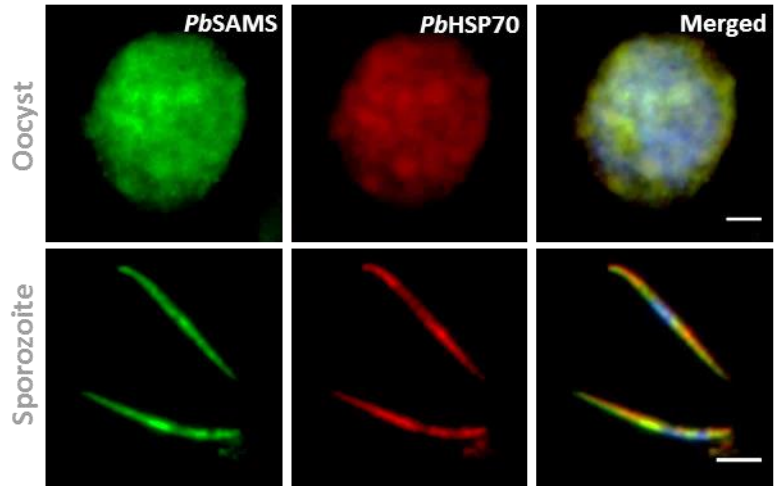

Figure 4.6– *Pb*SAMS localises to parasite cytosol during the mosquito-stages: oocysts and sporozoites. Widefield microscopy images of *Pb*SAMS-GFP oocysts and sporozoites, analysed at day 12 and 21 post infection, respectively. *Pb*SAMS localisation was assessed using the anti-GFP antibody and *Pb*HSP70 was used to stain *Plasmodium* cytosol. DAPI was used for nuclei staining. Scale bar - 5 µm

Extending the localisation studies to the exo-erythrocytic form of the parasite (liver-stage of parasite development), *Pb*SAMS-GFP-infected HepG2 hepatoma cells were fixed at developmentally relevant time points and an IFA was performed. Fluorescence microscopy analysis upon staining with the anti-GFP antibody showed that, similarly to the mosquito-stage, *Pb*SAMS co-localises with the *Pb*HSP70 (Figure 4.7). Altogether, these data show that *Pb*SAMS enzyme is expressed throughout all stages of *Plasmodium* life cycle, localising to the parasite cytosol.

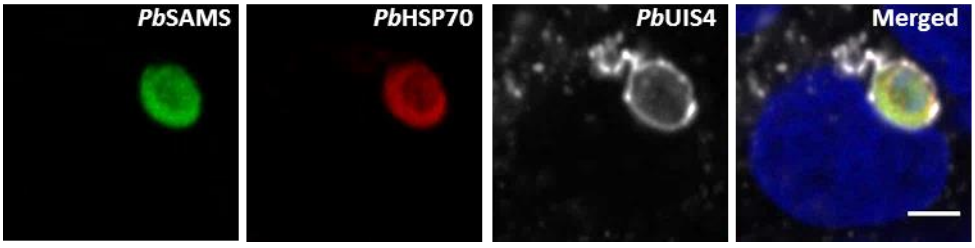

Figure 4.7– *Pb*SAMS localises to parasite cytosol during the liver-stage of the development. Widefield microscopy images of *Pb*SAMS-GFP parasites developing inside HepG2 cells. *Pb*SAMS localisation was assessed at 24 hpi using the anti-GFP antibody. *Pb*HSP70 was used to stain *Plasmodium* cytosol and DAPI was used for nuclei staining. Scale bar - 5 µm

2. Effect of *Pbsams* knockdown in *Plasmodium* survival and development

Deletion of genes that are essential for *Plasmodium* development during the blood-stage inescapably results in parasite death, hindering the characterization of the desired gene. To study the importance of *Pb*SAMS throughout the life cycle we tried to knockout *Pbsams* gene in three independent transfections. *Pbsams* was refractory to genetic deletion in blood-stage parasites, suggesting that has an essential role during that stage of development. In order to overcome this limitation we recurred to a conditional knockdown strategy by fusion of a destabilising domain system (DD) to the SAMS protein.

Destabilising Domain (DD) strategy

The working principle of this technique involves a chemical-genetic approach that allows the reversible expression of a protein of interest by genetic fusion to a destabilising domain (DD). The destabilising domain is constituted by mutants of *E.coli* dihydrofolate reductase (ecDHFR) and was engineered to be degraded. This DD is inherently unstable and in the absence of a stabilising ligand, the entire fusion protein is misfolded and degraded by the proteasome. Despite that, in the presence of the small-molecule ligand trimethoprim (TMP) the domain together with the entire fusion protein is stabilised, without affecting the target protein structure and function[52,53]. In this study, we worked with *Pb*SAMS-DD parasite lines previously obtained from two independent clonal lines. The desired construct containing the target gene fused to the DD, which is attached to the hemagglutinin (HA) tag, was introduced into *P. berghei* genome using a single crossover transfection strategy. After transfection, infected mice were treated with pyrimethamine to select a pure transgenic parasite population and with TMP for stabilisation of the SAMS-HA-DD fusion protein (hereafter referred to as SAMS-DD; Figure 4.8 A). Confirmation of the integration of the transgenic locus containing the 3'-HA-DD into the desired genome loci and confirmation of WT parasites removal was performed by genotyping PCR (Figure 4.8 B). Following the successful generation of the cloned transgenic *P. berghei* lines expressing the *Pb*SAMS-DD fusion protein, we analysed *Pb*SAMS role in *P. berghei* growth and development during the different stages of the life cycle.

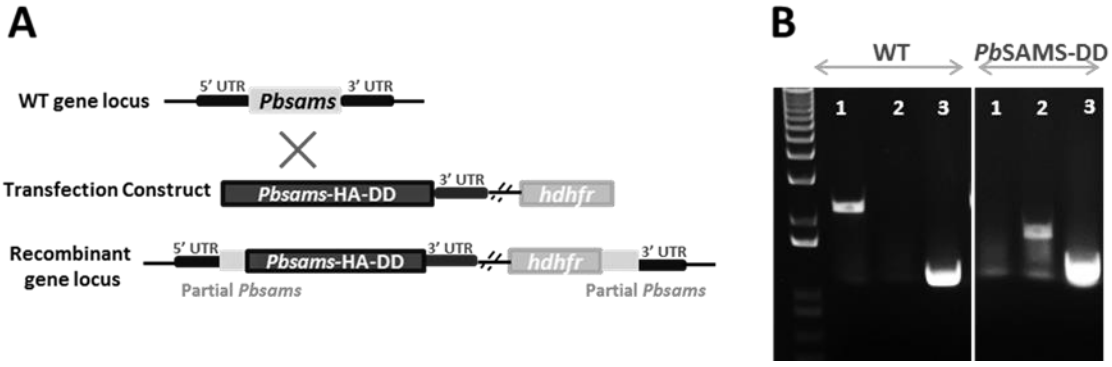

Figure 4.8 – *Pbsams* gene is essential during the blood-stage of infection hindering gene deletion. (A) Schematic representation of the Destabilising Domain strategy (DD) for *Pbsams* knockdown by single crossover recombination. **(B)** Genotyping PCR confirming a clonal population of *Pb*SAMS-DD parasites. Lane 1: detection of WT *Pbsams* locus; Lane 2: detection of knockdown integration locus; Lane 3: DNA quality control

2.1 SAMS is essential during the blood-stage development of *Plasmodium*

To assess the effect of *Pb*SAMS knockdown through destabilisation of the SAMS-DD protein in the outcome of the infection, Balb/c and C57BL6/J mice were infected through *i.v.* injection of 10^3 *Pb*SAMS-DD sporozoites and parasitemia was followed daily. It has been described that a successful DD-stabilisation by TMP treatment in mouse tissue requires, at least, a 3-week treatment[52]. Thus, stabilisation of the *Pb*SAMS-DD protein was achieved by pre-treating mice with TMP, 3 weeks before the infection until the end of the experiment. Balb/c mice do not develop severe malaria associated syndrome and succumb at very high parasitemia levels, allowing the study of *Pb*SAMS importance during a chronic infection. In contrast, C57BL6/J mice allowed the evaluation of the effect of *Pb*SAMS destabilisation in the onset of the severe disease syndrome, cerebral malaria, and survival. The length of the pre-patent period – time between sporozoite inoculation and the appearance of parasites in the blood – directly reflects the duration of the liver-stage and the number of infective merozoites produced. Results obtained suggest that *Pb*SAMS-DD destabilisation has no effect in parasites development during the liver-stage. This is evidenced by the appearance of both control (stabilised SAMS-DD; + TMP) and knockdown (destabilised SAMS-DD; - TMP) parasites in the bloodstream at the same day post infection - day 4 post infection, both in Balb/c and C57BL6/J mice, similarly to the WT parasite (Figure 4.9 A, 4.9 B).

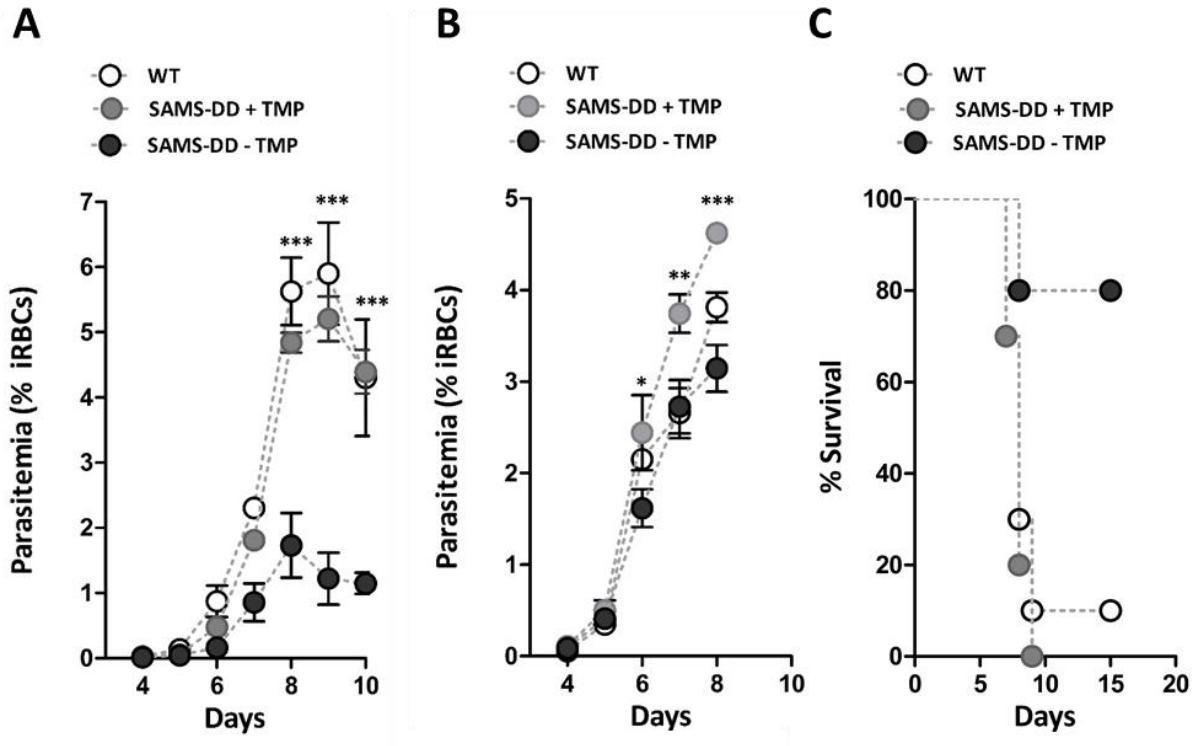

Figure 4.9 – Blood-stage parasites rely on *Pb*SAMS protein as its absence results in a developmental impairment.
(A,B) Progression of parasitemia in Balb/c **(A)** and C57BL/6 **(B)** mice following infection (*i.v.*) with 10^3 WT *P.*berghei or *Pb*SAMS-DD sporozoites, in the presence or absence of the stabilization ligand TMP. Parasitemia was determined by counting infected erythrocytes in Giemsa stained blood smears. **(C)** Survival of C57BL/6J mice infected *i.v.* with 10^3 *Pb*SAMS-DD sporozoites in the presence or absence of the stabilization ligand TMP. Error bars represent the SEM. Statistical significance was determined by the Mann Whitney U test, **p* < 0.05, ***p*<0.01, ***p<0.001. N=3

The results obtained show that *Pb*SAMS-DD destabilisation by TMP removal leads to a decrease in blood parasitemias both in Balb/c and C57BL6/J mice, although the difference is more pronounced in Balb/c mice. By contrast, mice infected with *Pb*SAMS-DD in the presence of TMP therefore leading to protein stabilisation, develop a parasitemia comparable to that of mice infected with WT parasites (Figure 4.9 A, B).

Analysis of survival curves show that while all mice infected with *Pb*SAMS-DD sporozoites in the presence of TMP died between day 8 and 9 post infection of cerebral malaria (CM), 8 out of 10 mice infected with *Pb*SAMS knockdown parasites (- TMP) survived. Thus, *Pb*SAMS destabilisation has an effect in the outcome of the infection resulting in a lower parasitemia and also in survival, providing some protection against this severe neurological complication (Figure 4.9 C). For all experiments performed with Balb/c mice, confirmation of protein stabilisation/destabilisation was performed by Immuno-blotting. Whole parasite extracts were obtained from blood of infected Balb/c mice collected 10 days post infection. *Pb*SAMS-HA-DD levels were detected using the anti-HA antibody. The *Pb*Bip was used as a loading control to normalize *Pb*SAMS-DD levels and, like this, evaluate destabilisation levels. Destabilisation by TMP removal steeply reduced the levels of *Pb*SAMS protein, suggesting that it is being degraded (Figure 4.10). These results obtained explain and reinforce the results obtained above, being in accordance with the marked differences obtained in parasitemia during the blood-stage of infection. This suggests that *P. berghei* relies on *Pb*SAMS to successfully complete its development during the blood-stage of infection.

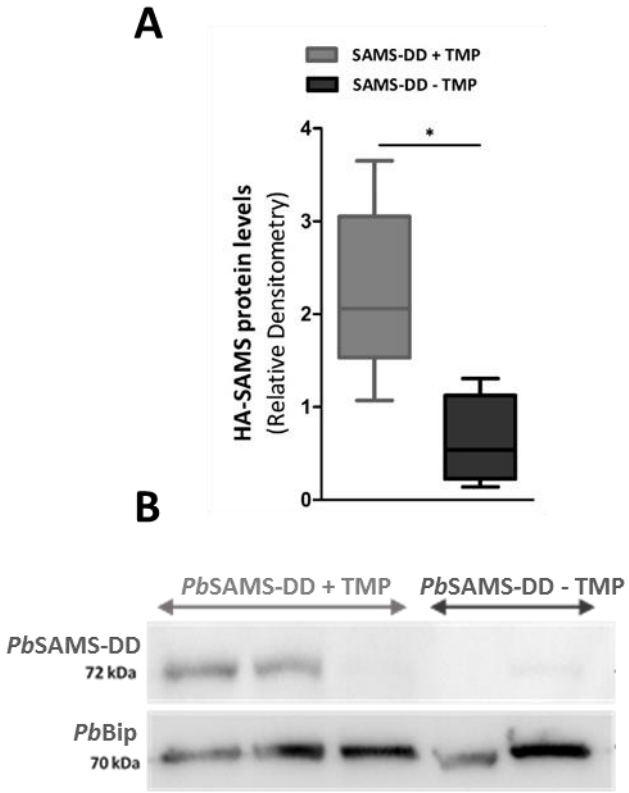

Figure 4.10 – Removal of TMP leads to *Pb*SAMS-DD destabilisation, resulting in a decrease in *Pb*SAMS expression levels. (A) Quantification of *Pbsams* knockdown in destabilised *Pb*SAMS with respect to the stabilised *Pb*SAMS protein using the HA antibody. Band quantification intensity was performed using the ImageJ software. *Pb*Bip intensity levels were used for normalization. Error bars represent SEM. Statistical significance was determined by the Mann Whitney U test, *p<0.05. **(B)** Representative picture of immuno-blotting analysis of *Pb*SAMS-DD destabilisation in whole parasite pellet extracts from mice-infected blood. The monoclonal mouse anti-HA antibody was employed to detect the HA-tagged *Pb*SAMS-DD fusion protein.

2.2 SAMS is not essential during the mosquito-stage development of *Plasmodium*

To assess the effect of *Pb*SAMS knockdown in oocysts and sporozoites development, *Anopheles* stephensi mosquitoes were infected with WT or *Pb*SAMS-DD parasites either in the presence (SAMS-DD + TMP) or absence (SAMS-DD - TMP) of the stabilising ligand. Stabilisation within the mosquito vector was assured by supplementing mosquito food with TMP (0.50 mg /mL). At day 12 post mosquito infection, midguts were dissected and oocysts were stained with mercurochrome. Oocyst number and size was assessed by light microscopy. Salivary glands were dissected 21 days post infection and the number of sporozoites obtained per mosquito was assessed. Destabilisation of *Pb*SAMS protein by TMP removal did not result in any statistically significant difference in both the size and number of knockdown (- TMP) *Pb*SAMS-DD oocysts, when comparing to the control (+ TMP) counterpart (Figure 4.11 A, 4.11 C). Sporozoites number obtained for both control (+ TMP) and knockdown (- TMP) *Pb*SAMS-DD parasites are also not significantly different, suggesting that this protein is not essential for *Plasmodium* successful development within the mosquito vector as both oocyst number and size and sporozoites number are comparable to WT parasites (Figure 4.11 B). Similarly to the blood-stage of infection, levels of protein stabilisation were assessed by immuno-blotting however, the low abundance of parasite material did not allowed the confirmation of protein stabilisation.

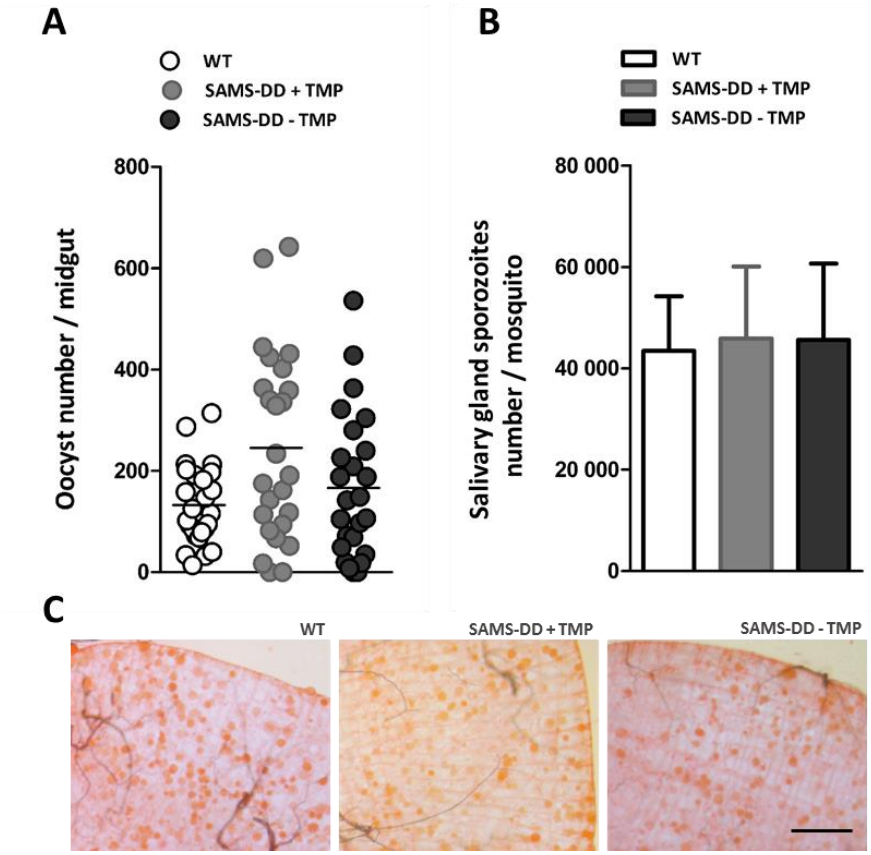

Figure 4.11 –*Pbsams* knockdown has no effect in *Pb*SAMS-DD oocysts and sporozoites development in *A. Stephensi* mosquitoes. (A) Quantification of oocyst number in WT- and *Pb*SAMS-DD-infected mosquito midguts dissected at day 12 post mosquito infection and stained with mercurochrome, by light microscopy. Each symbol represents an individual mosquito midgut. Horizontal lines represent the mean number of oocysts. **(B)** Quantification of sporozoites in WT- and *Pb*SAMS-DD-infected salivary glands dissected 21 days post mosquito infection. Error bars represent the SEM. **(C)** Representative image of oocysts size in mosquito midguts infected with WT (left panel) or *Pb*SAMS-DD parasites, on stabilisation (central panel) and destabilisation (right panel) of *Pb*SAMS. Scale bar – 100 µm. Statistical significance was determined by the Mann Whitney U test, $p>0.05$. N=3

2.3 SAMS is not essential during the liver-stage development of *Plasmodium*

The effect of *Pb*SAMS knockdown during *Plasmodium* liver-stage of infection was assessed *in vitro,* in HepG2 cells. Cells were infected with *Pb*SAMS-DD sporozoites and treated with increasing concentrations of the stabilising ligand, TMP (0.5 and 5 µM). Exoerythrocytic forms (EEFs) development was assessed by IFA using the anti-HA antibody. The results show that *Pb*SAMS destabilisation does not affect infection rate, as evidenced by the similar number of EFFs produced by both control (+ TMP) and knockdown (- TMP) *Pb*SAMS-DD parasites (Figure 3.12 A). Furthermore, results also show that parasite replication is not altered in the absence of *Pb*SAMS, as the size of EEFs is similar (Figure 4.12 B).

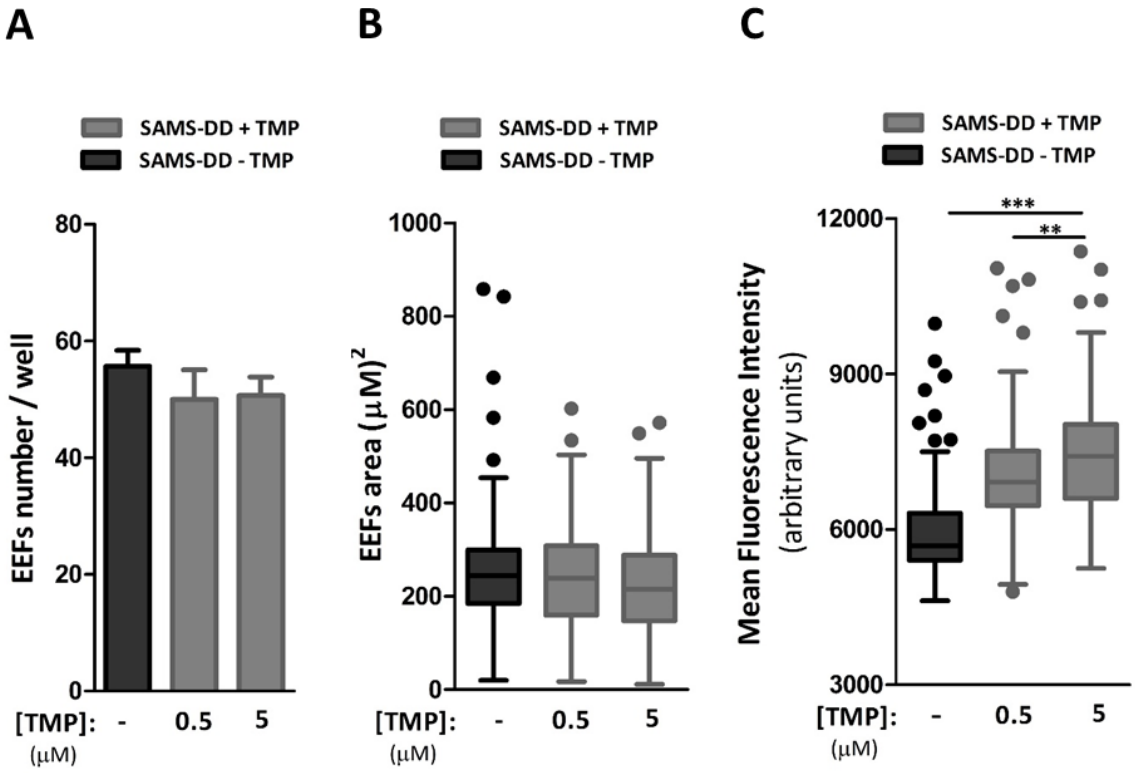

Figure 4.12 – *Pb*SAMS destabilisation by TMP removal results in a decrease of protein levels without affecting liver-stage parasites replication and growth, *in vitro*. (A-B) Quantification of the **(A)** number and **(B)** size of *Pb*SAMS-DD EEFs under different concentrations of the stabilising ligand TMP, analysed at 48 hours post infection of HepG2 cells, assessed by confocal microscopy. **(C)** Quantification of SAMS-DD levels, in EEFs developing under different TMP concentrations, by measuring HA fluorescence intensity. Horizontal lines represent mean fluorescence intensity for the HA-tagged *Pb*SAMS protein for each group. Error bars represent the SEM. Statistical significance was determined by the Mann Whitney U test, **p<0.01, *** p<0.001. N=3

The stabilisation of *Pb*SAMS by TMP treatment was analysed by IFA using the anti-HA antibody and fluorescence intensity was quantified, allowing the measurement of *Pb*SAMS levels and subsequently, of the extent of *Pbsams* knockdown. Data obtained show that *Pb*SAMS levels are lower in the absence of TMP, suggesting that *Pb*SAMS is being degraded (Figures 4.12 C, 4.13). In addition, results also suggest that TMP stabilises *Pb*SAMS in a dose-dependent manner, as evidenced by an increased in fluorescence intensity when higher concentrations of TMP are added (Figure 4.12 C).

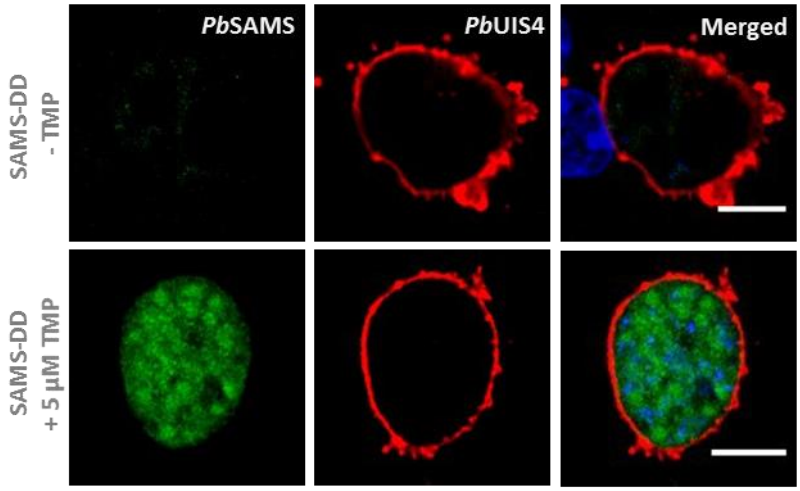

Figure 4.13 – TMP removal results in a decrease of *Pb*SAMS protein levels during *in vitro* liver-stage infection. Confocal images of *Pb*SAMS-DD parasites developing in HepG2 cells in the presence or absence of the stabilizing ligand (TMP). *Pb*SAMS-DD was detected using the anti-HA antibody. The anti-UIS4 antibody was used as parasitophorous vacuole membrane marker and DAPI was used for nuclei staining. Scale bar - 5 µm

To assess the effect of *Pb*SAMS knockdown in *Plasmodium* growth and replication during hepatic development *in vivo,* C57BL/6 mice were infected with 5 x 10^4 *Pb*SAMS-DD sporozoites either in the presence (+ TMP) or absence (- TMP) of the stabilising ligand. Thus, to ensure *Pb*SAMS stabilisation during liver infection, mice were pre-treated 3 weeks before infection with TMP in drinking water (0.25mg/mL). Mice were kept under treatment until the end of the experiment. Livers were harvested 48 hours after sporozoites infection (*i.v.*) and total RNA was extracted and converted into cDNA. Parasite load in the liver was quantified by RT-qPCR using specific primers to *P. berghei* 18S rRNA. Mouse *hprt* expression levels were used as housekeeping gene for normalization.

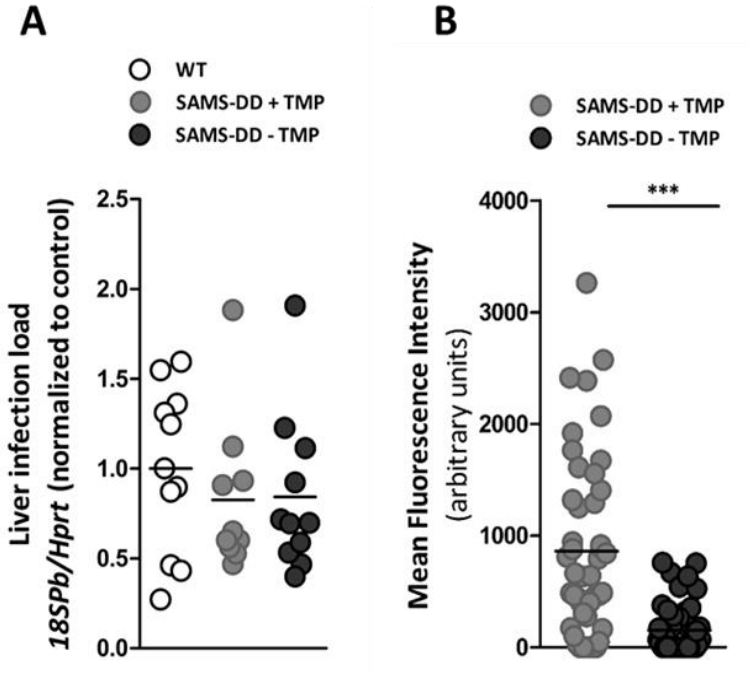

Figure 4.14 – *Pb*SAMS destabilisation by TMP removal decreases protein levels without affecting liver infection, *in vivo*. (A) Parasite liver load in C57BL/6J mice infected with WT or *Pb*SAMS-DD sporozoites, and treated or not with TMP. Liver load was measured 48hpi by RT-qPCR of *P. berghei* 18S rRNA and normalized to mouse *hprt* housekeeping gene. Each symbol represents an individual mouse. **(B)** Quantification of SAMS-DD levels, in EEFs developing in the presence or absence of TMP, by measuring HA fluorescence intensity Horizontal lines represent the mean fluorescence intensity for the HA-tagged *Pb*SAMS protein for each group. Error bars represent SEM. Statistical significance was determined by the Mann Whitney U test, *** p<0.001.

Results obtained show that destabilisation of *Pb*SAMS protein by TMP removal has no effect in parasite liver load, when compared to the stabilised counterpart both comparable with the WT parasite (Figure 4.14 A), suggesting that *Pb*SAMS does not possess a key role during *Plasmodium* hepatic infection.

Stabilisation levels of *Pb*SAMS by TMP treatment, in the same livers, were quantified by IFA. A liver lobe was fixed and subsequently sliced into 40-µM sections. HA-tagged *Pb*SAMS levels were detected and quantified using the anti-HA antibody. The results obtained show that *Pb*SAMS is stabilised when TMP is added, as evidenced by a marked increase in fluorescence intensity for the HA staining when compared to fluorescence levels obtained for the destabilised (- TMP) condition (Figures 4.14 B, 4.15).

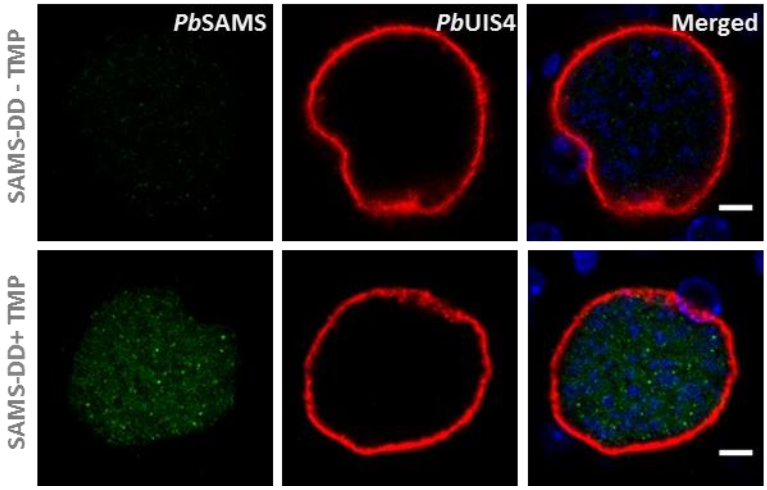

Figure 4.15 - TMP removal results in a decrease of *Pb*SAMS protein levels during *in vivo* liver-stage infection. Confocal images of *Pb*SAMS-DD parasites developing in livers of mice treated or not withTMP. *Pb*SAMS-DD was detected using the anti-HA antibody. The anti-UIS4 antibody was used as parasitophorous vacuole membrane marker and DAPI was used for nuclei staining. Scale bar - 5 µm

V. DISCUSSION AND FUTURE DIRECTIONS

All parasitic infections rely on robust metabolic interactions between the parasite and its host. This metabolic interplay between host and parasite is the basis for the establishment of a successful infection as far as parasites take advantage of host nutrients to ensure their own growth and replication. At the same time the host needs to handle with and struggle against, toxins and tissue damage that arise from infection in order to maintain homeostasis. As a rapidly growing organism, *Plasmodium* heavily depends on this metabolic exchanges with the host to survive. As such, malaria parasites developed mechanisms to exploit host nutrients through the modulation of their metabolic network[54]. During the blood-stage of infection *Plasmodium* obtains methionine through haemoglobin degradation and simultaneous induction of NPPs in the host cell surface improving the transport and therefore, the uptake of this vital amino acid[36]. However, the mechanisms by which *Plasmodium* modulates host cells and how much the host cell contributes to parasites survival and to the establishment of a successful infection during the liver- and the mosquito-stage, is not described in detail. Since the hepatic stage of *Plasmodium* development is silent, occurring before the onset of the pathology, it is a promisor target for the development of an intervention strategy. Despite the availability of several therapeutic and prophylactic drugs, malaria still remains a major health concern. The appearance of resistance against the available drugs coupled with a scarce understanding of *Plasmodium* biology and in its interactions with the host, make this goal harder to reach. Thus, the study and characterization of novel drug targets in *Plasmodium* parasites are critical to reduce the burden of malaria worldwide.

The first step of methionine cycle is the synthesis of SAMe, the major biological methyl donor in all living cells. Given the unique role of the hepatocyte in SAMe synthesis - about 80% of SAMe is synthesized in the mammalian liver - and the importance of SAMe dependent transmethylation reactions in cell growth and replication we hypothesized that *Plasmodium*, while replicating inside hepatocytes, ensures its nutritional demands by scavenging this metabolite from the host cell. Inside hepatocytes *Plasmodium* undergoes an exponential replicative process, achieving one of the fastest growth rate among eukaryotic cells – each parasite develops into 10 to 30 000 merozoites according to *Plasmodium* spp[55,56]. To fulfill their metabolic requirements and to withstand this fast growth, parasites must scavenge nutrients from the host cell, including the essential metabolite SAMe. *Plasmodium* tropism to hepatocytes coupled with the obligatory liver-stage of infection suggests that the host hepatocyte plays a key role in *Plasmodium* development, reflecting the unique metabolic properties of mammalian hepatocytes.

Data herein described show that during the blood-stage of infection *Plasmodium* growth and replication is influenced by the metabolism of methionine. We show that, *Pb*SAMS knockdown parasites (*Pb*SAMS-DD – TMP) and consequently unable to synthesize SAMe, undergo a developmental impairment that results in lower parasitemia in *Pb*SAMS-DD - TMP-infected mice. These results suggest a role for methylation or polyamines in *Plasmodium* development and in the establishment of a successful infection during the blood-stage of infection.

The lower parasitemia observed may be due to *Plasmodium* inability to synthesize endogenous SAMe coupled with either: 1) an insufficient supply of SAMe by the host cell; or 2) *Plasmodium* inability to take it up from the host cell. Still, a small number of parasites can proceed with blood-stage infection suggesting that the low amounts of SAMe produced by the host erythrocyte together with the fact that the DD system employed for the *Pb*SAMS inducible knockdown strategy is leaky, may be sufficient to support the growth of a reduced number of parasites. Notably, our data show that while *Pb*SAMS-dependent SAMe synthesis is essential for blood-stages of infection, it is not essential throughout other stages of *P. berghei* life cycle. During the liver- and in the mosquito-stage *Plasmodium* development is not affected by the lack of SAMS and consequently, by the low levels of SAMe, suggesting that parasites offset the lack of SAMe synthesis through its uptake from the host. In fact, this is quite plausible given that the hepatocyte is a privileged cell in terms of methionine metabolism and SAMe production. The exclusive expression of MAT I/III enzyme with kinetic properties that allow for the production of virtually unlimited amounts of SAMe[19], may constitute the ideal environment for *Plasmodium* intense intra-hepatic replication. From the therapeutic point of view the methionine metabolic pathway, as an essential metabolic via during the blood-stage of development, may constitute a new antimalarial target. The treatment with an inhibitor of *Pb*SAMS, consequently leading to inhibition of SAMe synthesis may result in parasite death, opening a new line of research toward the development of novel antimalarial therapeutics.

Methionine is also the precursor of the antioxidant glutathione through the transsulfuration pathway. *Plasmodium* infection leads to an increased oxidative stress both in the mammalian host and in the mosquito vector. To ensure the maintenance of a redox equilibrium, *Plasmodium* evolved antioxidant mechanisms such as the GSH which constitutes the primary line of defense against ROS. Thus, we expected that parasites lacking GS (*Pbgs*$^{-}$) due to an impaired response to the external oxidative stress, would face a major difficulty to grow and survive in pro-oxidative environments - such as the infected erythrocyte of the mammalian host and the mosquito vector. Our results showed that in the absence of the antioxidant GSH, *Pbgs*$^{-}$ parasites survived during the blood-stage of the infection, suggesting that *P. berghei* do not depend on the *de novo* synthesis of GSH to complete a successful asexual development. This observation suggests that *P. berghei* overcomes the lack of GSH through scavenging the host metabolite, possibly from the host erythrocyte and further transport to the food vacuole, via endocytic vesicles that contain haemoglobin[50]. While in the blood-stage GS deletion has a minor effect in parasites growth and survival in the mosquito vector it has a dramatic effect. During the mosquito-stage ROS levels are known to increase within the mosquito midgut as a consequence of: 1) the innate immune response of the vector to *Plasmodium* infection and also due to 2) *Plasmodium* switching from glycolysis to oxidative phosphorylation to obtain energy, resulting in an increased mitochondrial activity and consequently, in the production of large amounts of ROS. Additionally, oocysts development occurs extracellularly which results in an increased vulnerability to these reactive species during that stage of development.

Results obtained suggest that a fully functional GSH antioxidant system is essential, as parasites lacking GS and therefore, unable to synthesize GSH may not be able to deal with this increased oxidative stress.[57] Thus, an increased demand for this tripeptide during that stage of development coupled with an insufficient supply of GSH by the host results in parasite death during oocysts development. In fact, the majority of anti-malarial drug therapies against blood-stages, namely chloroquine, primaquine and derivates of artemisin, promote oxidation through mechanisms that induce the production of free radicals. Increasing ROS levels within the mosquito midgut and blocking *Plasmodium* GSH biosynthetic pathway may constitute a promising tool to prevent parasite survival and spreading as: 1) *Pbgs*$^{-}$ parasites are not able to develop into infectious sporozoites, preventing the transmission from the mosquito vector to the mammalian host; and 2) the developmental arrest at the oocyst stage is not rescued by mosquito blood feeding on mice. As an essential via during the mosquito-stage, treatment with an irreversible inhibitor of *P. berghei* GSH synthesis may be a promising approach to develop novel transmission-blocking strategies. Due to its essentiality during the mosquito-stage, characterization of *P. berghei* GS enzyme during the liver-stage has been specially challenging. The inability to produce *Pbgs*$^{-}$ sporozoites compromised the transmission from the mosquito vector to the mammalian, preventing further characterization of the role of GS during the liver-stage of development. To overcome this limitation further studies are needed focusing either to 1) inhibition of *Plasmodium* switching from glycolysis to oxidative phosphorylation during the mosquito-stage of development, ensuring a low level of ROS; or 2) generation of *Pbgs*-lacking parasites through inducible knockdown which will allow us to specifically silence our gene of interest after sporozoites production.

Altogether, the results obtained in this study identify potentially relevant targets for the development of antimalarial drugs pointing either to the blood-stage (*Pb*SAMS), preventing the progression of disease and further relapsing (via *P. vivax*) and to the mosquito-stage (*Pb*GS), preventing the differentiation of sexual parasites into thousands of infective sporozoites, hindering transmission from the vector to the mammalian host with potential to substantially reduce the spread of the disease.

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VII. ANNEXES

1. Primers List and Sequence:

Supplementary table 1 - List of primers used in PCR and RT-qPCR reactions

Target gene	Application	Sequence
Pb gs **(WT locus)**	Genotyping PCR – forward primer	ACCCCAAAGAGAAGGTGGACA
	Genotyping PCR – reverse primer	AGGAAAATCGCCATTCCACAAGCA
Pb gs **(integration locus)**	Genotyping PCR – forward primer	AGGAAAATCGCCATTCCACAAGCA
	Genotyping PCR – reverse primer	CTTTGGTGACAGATACTAC
Pb sams **(WT locus)**	Genotyping PCR – forward primer	TAGGTACCGAGGAAATTTTCTATTTACTTCG
	Genotyping PCR – reverse primer	ATGCGGCCGCCAACTAATAAAATCCAGGAAATA
Pb sams **(integration locus)**	Genotyping PCR – forward primer	TAGGTACCGAGGAAATTTTCTATTTACTTCG
	Genotyping PCR – reverse primer	TAGCGGCCGCGGTCATGCGTAG
Pb sams **(DNA quality control)**	Genotyping PCR – forward primer	TAGGTACCGAGGAAATTTTCTATTTACTTCG
	Genotyping PCR – reverse primer	TAGGGCCCATTTTTTAAAACATTTTTTTCGTG
Mouse hprt	RT - qPCR – forward	TTTGCTGACCTGCTGGATTAC
	RT - qPCR – reverse	CAAGACATTCTTTCCAGTTAAAGTTG
Pb**18S rRNA**	RT – qPCr – forward	AAGCATTAAATAAAGCGAATACATCCTTAC
	RT – qPCR – reverse	GGAGATTGGTTTTGACGTTTATGTG
Pb sams **(PBANKA_082310)**	RT – qPCr – forward	AGTTGATCGTTCCGCAGCTT
	RT – qPCR – reverse	TTAGCGACACTGGATTGGCA
Pb **serine tRNA ligase (PBANKA_0615400)**	RT – qPCr – forward	ATTGCTCAACCTTATCAAACTG
	RT – qPCR – reverse	AGCCACATCTGAACAACCG
Pb a**denylosuccinate lyase (PBANKA_0304300)**	RT – qPCr – forward	TGCCTCATAAAATAA ACCCA
	RT – qPCR – reverse	TCGTAAAACTGTTGAATCGG